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ASSESSMENT OF VIABILITY OF PROBIOTIC BACTERIA IN NON DAIRY FOOD MATRICES UNDER REFRIGERATION STORAGE

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ASSESSMENT OF VIABILITY OF PROBIOTIC BACTERIA IN NON DAIRY FOOD MATRICES UNDER REFRIGERATION STORAGE

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ABSTRACT

Functional foods serve nutrients and physiologically active components for a healthy living. In most countries, the products related to having a healthy gut dominate the functional foods market. These products are prebiotics, probiotics and synbiotics. Concerning the health benefits, various probiotics are introduced to food matrices. Though dairy is the ideal food matrix for probiotics, the trend of non-dairy probiotics is growing among consumers.

This study included two research objectives: surveying the commercial probiotic containing products for probiotic level and developing a practical home-made probiotic fermentation process. The first part of this study was mainly based on assessing viability of probiotic bacteria in non-dairy matrices. Six commercially available probiotics products were tested to verify the manufacturer compliance with the claimed amount on the labels under refrigeration storage for the recommended shelf life. Five products were non-dairy matrices including both liquid and solid matrices. One was a dairy substrate. Viability was assessed by plate counting and flowcytometry. Flowcytometry analysis followed by staining with Live/Dead BacLight Viability Kit, did not provide consistent acceptable counts. Therefore viable counts by plate counting were compared with the manufacturer claims. All products were in the acceptable range for therapeutic minimum (10^6-10^8 CFU/ml or CFU/g). However, four products had significantly ($p<0.05$) lower counts than the manufacturer claims. When the stability during the refrigeration temperatures is concerned, fruit juice (blackberry - pomegranate) and chocolate were more stable food matrices.

The second part of this study was based on developing a novel food product based on fermented coconut water and oatmeal. For the safety, commercial coconut water and oatmeal were used; *Lactobacillus plantarum* Lp 115-400B, which is a proven probiotic, was used as the starter culture. The evidence of some unpublished studies, that revealed fermented coconut water possesses some healing power of unusual bowel movements led us to use coconut water as the fermentation substrate. Oatmeal-coconut water matrix was inoculated with 10^7 CFU/g of *L. plantarum* to have the novel mixture. Fermentation kinetics was obtained by evaluating the viability at 24, 32, 27, 42 and 47 ºC at 2h intervals using Arrhenius equation. For the preparation of the fermented product, 27 ºC was used with 10h fermentation (n=3). The experiment was designed as a split plot block design. The fermented product was refrigerated (4±1ºC) and viability of *L. plantarum* was enumerated weekly by plate counting. The recommended minimum daily dose of inulin (1g) was added to the same matrix and viability was compared with the samples without inulin (n=3). Both pH and total acidity of the matrix were also monitored weekly. The apparent viscosity was measured on the production day (day 0) and on the last test day (day 49). The inulin dose used in this study does not affect the viability of selected *L.plantarum* strain within this matrix. The shelf life of the novel food matrix was determined by counting the days taken to reach 10^7 CFU/g. Shelf life was 7 weeks without inulin and with inulin it was 5 weeks. A significant reduction of pH was observed at the end of the considered shelf life. The apparent viscosity of the product did not change significantly ($p>0.05$) after the fermentation.

In conclusion, out of six commercial products, five products do not deliver the claimed probiotic counts by the manufacturer at the end of the shelf life. The fermented novel food matrix sustains *L. plantarum* for 7 weeks at successful levels under refrigeration.

DEDICATION

I dedicate this work to my parents, my beloved husband and son, my sister and brother. This work would have not been possible without their love, support and encouragement.

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LIST OF ABBREVIATIONS

ADA = American Dietetic Association

CFU = Colony Forming Units

EPS = Exopolysaccharides

FAO = Food and Agriculture Organization of the United Nations

FCM = Flowcytometry

FDA = Food and Drug Administration

FISH = Fluorescence In Situ Hybridization

FOSHU = Foods for Specified Health Use

FSC = Forward Scatter

GI = Gastrointestinal

GRAS = Generally Recognized As Safe

IFT = Institute of Food Technologists

Ig = Immunoglobulin

LAB = Lactic Acid Bacteria

MPN = Most Probable Number

MRS = de Man Rogosa and Sharpe

NASBA = Nucleic Acid Sequence Based Amplification

RT-PCR = Reverse Transcriptase Polymerase Chain Reaction

SCFA = Short Chain Fatty Acids

SSC = Side Scatter

Th = T helper cells

TNF = Tumor Necrosis Factor

VBNC = Viable But Non Culturable

Y & M = Yeast and Mold

WHO = World Health Organization

INTRODUCTION

According to the World Health Organization and the Food and Agriculture Organization of the United Nations (WHO/FAO) the definition of probiotics is "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host"(2001). Due to growing interest on health benefits, probiotic bacteria are included within various novel food matrices.

To gain the expected health benefits as WHO/FAO describes, “live probiotic bacteria” must be consumed. Many authors consider the number of cells required to affect the gastrointestinal environment is between 10^6 to 10^8 CFU/ml or CFU/g of the food item. This accepted dose is named as “the therapeutic minimum” (Shah, 2000; Lahtinen *et al*, 2006; Vasiljevic and Shah, 2008; Shafiee *et al*, 2010). However, a probiotic product is considered as functional only if it contains 10^7 CFU/ml at the time of consumption (Charalampopoulos *et al*, 2002). In selecting probiotic bacteria for industrial manufacturing processes safety, functional, and technological characteristics should be considered. Functional properties include the viability of cells, stability of the cells within a food matrix, persistence activity in gastrointestinal tract, species and strain characteristics, daily dose, fermentation technology, storage conditions, and availability of prebiotics (Georgieva *et al*, 2009; Mattila-Sandholm, 2002; Ranadheera *et al*, 2010).

Prebiotics are defined as “non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth and/or activity of one or a limited number of microbes in the colon resulting in documented health benefits” (Forssten *et al*, 2011; de Vriese and Marteau, 2007). Prebiotics are added in functional probiotic food

formulation to increase the viability of probiotics and shelf life of the product. Prebiotics can be added separately or sometimes the food matrix itself has components with prebiotic properties such as beta glucan in oats. Hence, using such food matrices for fermentation will be more effective to deliver probiotics than the capsules (Sanders and Marco, 2010). Mostly, *Bifidobacteria* growth is promoted by prebiotics and products that keep the combination of probiotics and prebiotics are called “synbiotics”.

The probiotic bacteria, which are used as a food ingredient industrially, can be obtained as freeze dried cultures. The probiotics undergo a great stress under processing and storage conditions of the freeze-dried cultures and also inside the food matrix after manufacturing process. Survival of probiotics in a suitable food matrix is also affected by a range of factors including pH, post-acidification (during storage) in fermented products, hydrogen peroxide production, oxygen toxicity (oxygen permeation through packaging), storage temperatures, stability in dried or frozen form and lack of proteases (Kailasapathty, 2002). All these factors raise the stress on the microorganisms in keeping their physiological and biological functions or the functionality of cells within the matrix. The stress conditions may cause the probiotic cells “a sub-lethal injury” preventing the cell division (Kell *et al*, 1998; Oliver, 2005). Lactic acid bacteria (LAB) have defense mechanisms such as producing stress-induced proteins to regulate stress conditions and maintain the viability in food matrices (de Guchte *et al*, 2002). Under such conditions, cell could be non-culturable, but still alive. These non-growing surviving cells are called as “viable but non-culturable cells” or “VBNC”. Enumerating the viable cells in a probiotic food matrix may underestimate the actual functional probiotic count in

conventional counting on agar. Over 60 species of bacteria enter the VBNC status including human pathogens (Oliver, 2005).

For commercial manufacturing processes, starter cultures are selected that contain a large number of viable and uninjured cells. Mostly freeze-dried cultures of lactic acid bacteria, which contain over 10^{11} CFU/g, are used commercially (Mattila-Sandholm, 2002). Selecting a food matrix, which can carry a probiotic culture at effective levels, is a challenge. Most probiotic products are dairy-based since ancient times. Dairy is a very nutritious substrate, which satisfies the nutritional requirements of fastidious LAB. But, expanding trend of vegan lifestyles, the issues of lactose intolerance, and the demand for low-fat and low-cholesterol foods have created a growing demand for non-dairy probiotic products. Consequently, whole cereals, which contain prebiotic constituents in the bran, are getting more attention as potential substrates. While granola bars and oat containing beverages are available in the market, many other cereals such as wheat, barley and malt have also been tested experimentally as food matrices for probiotics by tracking the viability of the inoculated probiotics strains (Charalampopoulos *et al*, 2002).

For commercial probiotic products, it is very important to keep the stability of live microorganisms throughout the shelf life of the products. Though there are some opinions that even dead/inactive cells can act as probiotics, the viability is a key factor for starter culture (Isolauri *et al*, 2004). The carrier food item should contain the expected viable probiotic count till the "best before" date.

This thesis is based on the results of two studies. In study I, some commercial probiotic matrices were tested within the shelf-life during the refrigeration storage (4 ºC).

In study II, a new and inexpensive novel functional food matrix, which, is composed of two popular food substrates; oatmeal, green coconut water was prepared and inoculated with *L. plantarum* – Lp 115-400B.

The main goal of this project was to determine the viability of probiotic bacteria in non - dairy food matrices under refrigeration storage. The objectives were:

1. To assess the compliance of the manufacturers' claims and probiotic efficacy of different commercial probiotic matrices during the shelf life.
2. To study the fitness of coconut water and oat meal to sustain *L.plantarum* lp115-400B in a home-made probiotic product.
3. To monitor the survival of *L.plantarum* lp115-400B during processing and storage under refrigeration temperatures in oatmeal-coconut water food matrix.
4. To assess the contribution of a selected prebiotic on the growth of the probiotic bacterium in oatmeal-coconut water food matrix.
5. To examine the growth rate of the selected probiotic bacterium under different fermentation temperatures in oatmeal-coconut water food matrix.
6. To monitor the acidification by pH measurements and titrations and changes of rheology of the medium with oatmeal-coconut water.

LITERATURE REVIEW

Functional Foods

The term “functional food” was originated in Japan in the 1980s and this functional food concept obtained the legal status in 1991 by setting up “Foods for Specified Health Use” (FOSHU) regulatory system (Staton *et al*, 2001; Prado *et al*, 2008). The demand for functional foods was increased in recent years broadening the market for functional foods (Staton *et al*, 2001). Functional foods are designed foods with some modifications to be “functional” (Shah, 2007). There are numerous definitions for functional foods. The Institute of Food Technologists (IFT) expert report defines it as “foods and food components that provide a health benefit beyond basic nutrition” (IFT, 2005). This may include conventional foods, fortified, enriched or enhanced food and dietary supplements. The American Dietetic Association (ADA) defines functional foods as “Food, that includes whole foods and fortified, enriched or enhanced foods, have a potentially beneficial effect on health when consumed as part of a varied diet on a regular basis, at active levels.”

According to ADA there are four different functional food categories: conventional foods, modified foods, medical foods, and foods for special dietary use. Fruits and vegetables, which are rich in phytochemicals and yogurt that is rich in probiotics are some examples for conventional foods. Modified foods are functional foods that have enriched, fortified, or enhanced with bioactive components such as calcium-fortified milk and orange juice. Medical foods are formulas administered only under physician supervision for specific health problems. Foods for special dietary use such as gluten free products target specific health issues, but, a physician

recommendation is not required (Furguson, 2009). In 2008, Doyon and Labrecque gave a better definition for functional foods after considering 26 prevailing definitions, “a functional food is, or appears similar to, a conventional food. It is part of a standard diet and is consumed on a regular basis, in normal quantities. It has proven health benefits that reduce the risk of specific chronic diseases or beneficially affect target functions beyond its basic nutritional functions”. Under this definition, pills and capsules that are not similar in appearance to conventional foods are not considered as functional foods.

Because of consumer concerns for their gut health, many functional food items are entering the market places. These functional foods alter the microbial composition and quantity in gut and also the fermentation pattern of bacteria. Usually three basic ingredients are added to functional foods designed for gut health. They are living microorganisms (probiotics), non-digestible carbohydrates (prebiotics), and secondary plant metabolites, such as polyphenol compounds (Puupponen *et al*, 2003).
Probiotics is the fastest growing sector in the functional food market (Salmeron *et al*, 2009). According to the above definitions, a scientifically proven probiotic species can be used as food additives to produce functional foods (von Wright, 2005).

Concept of Modern Probiotics

Probiotics have been consumed by human beings in the form of fermented dairy products for a long time without a proper understanding of the health effects. Probiotic bacteria associated with fermentation are also referred to as "friendly bacteria" or "good bacteria”. Probiotics are live microorganisms that are similar to beneficial microorganisms found in the human gut.

Nowadays food products with these probiotics are abundant in the market. The isolated, safe microorganisms / probiotics are added as food ingredients and functional foods are produced. Using probiotics in fermentation is not a modern concept in food industry. Microbial fermentation is one of the oldest, economical, and practical technologies used in food preservation.

The concept of 'modern probiotics' was proposed for the first time by a Russian scientist, Elie Metchnikoff (Pasteur Institute, France) in 1907 (Ranadheera *et al*, 2010; Vasiljevic and Shah, 2008). Metchnikoff observed the longevity of life of the Bulgarian peasants due to consumption of fermented milk containing lactobacilli (Ranadheera *et al*, 2010; Kailasapathy and Chin, 2000). With this observation, he concluded that maintaining a healthy intestinal microflora is a secret for a healthy, long life (Ranadheera *et al*, 2010; Shah, 2007).

Contemporary with that, in 1906, a pediatrician, Henry Tissier observed the number of bacteria with a typical 'Y' shaped cell morphology were less in children with diarrhea than that of healthy children. With his observation he also concluded that, these bacteria play a role in maintaining the gut health (FAO/WHO, 2001).

Probiotic products are abundantly available in Europe and Japan. The USA is also paying more attention to probiotic products concerning being healthy with natural substances (Sanders, 1999). Though people consume probiotics as fermented products, nowadays probiotics are added as supplements (Ranadheera *et al*, 2010). Probiotics are delivered to gastrointestinal tract in several forms including probiotic foods, food ingredients, and dietary supplements. Probiotics are available as neutraceuticals in

capsules. Direct-fed microbials, which are used for animals, and genetically modified probiotics or designer probiotics, are also available forms of probiotics.

The most prominent probiotic microorganisms are Lactic acid bacteria, and *Bifidobacteria* species. Strains of *Lactobacillus plantarum, Lactobacillus acidophils, Lactobacillus rhamnosus, Lactobacillus reuteri, Lactobacillus johnsonii, Lactobacillus lactis, Lactobacillus casei*, *Lactobacillus paracasei, Lactobacillus delbrueckii* subsp *bulgaricus, Bifidobacterium lactis, Bifidobacterium infantis, Bifidobacterium longum,* and *Bifidobacterium brevis* are frequently used as commercial starter cultures (Lahtinen *et al*, 2006; Champagne and Gardner, 2005). In addition to those *Streptococcus thermophillus, Enterococcus francium, Pediococcus* and *Leuconostoc* species are also LAB, used as probiotics (Ranadheera *et al*, 2010). They inhabit in large intestine of warm blooded animals naturally. *Saccharomyces baulardii* is a yeast, which is considered as a probiotic and is being used commercially. The probiotics can be used as single or mixed cultures.

There are several requirements to be satisfied in order to be a probiotic bacterium. The strains used for humans should have a human origin and should show the benefits inside the human body, classified and well identified using internationally accepted methods, should not be pathogenic to human at all, and carry any antibiotic resistant genes (Saarela *et al*, 2000; FAO/ WHO, 2001). LAB can be isolated from many natural habitats. Though the strains available associated with plant materials do not have a human origin, they are still potentially good probiotics (Champagne and Gardner, 2005). To be active inside the human gut, probiotics should be acid and bile tolerant, adhere the

gut surfaces, show antagonistic effects on pathogens, such as *Helicobacter pylori, Salmonella* spp., *Listeria monocytogenes* and *Clostridium difficile*, show aniticarsinogenic and antimutagenic properties and stimulate gut associated mucosal membranes (Saarela *et al*, 2000). A bacterial strain is not considered as a probiotic until the health benefits are proven clinically (Reid, 2008).

Health Effects of Probiotics

Probiotics may exert many health effects associated with the gut though the mechanisms of action are not clearly known. The general public is more perceptive on health issues than older days. An increased in life expectancy, an increased in life-style related diseases and the high costs of health care persuade people for prevention from diseases. The microflora of the GI tract changes from time to time throughout the lifetime of human beings. The prominent flora of infants is *Bifidobacteria*. In adults, the prominent flora is *Bacteroides*. At the older age, B*ifidobacteria* count decreases and harmful bacteria such as coliforms, enterococci and *Clostridium perfringens* counts are higher compared to young adults (Lourens-Hattingh and Viljoen, 2001, Tiihonen *et al*, 2010). The altered microflora is due to impaired immunity, reduced consumption of fibers with diet, and using antibiotics frequently at older ages (Tiihonen *et al*, 2010). As a result the functional food market is growing yearly (Doyon and Labrecque, 2008).

Prevention and Reduction of diarrhea. The general public believes that fermented milk prevents intestinal infections. This disease preventing property is due to presence of LAB and Bifidobacteria in fermented milk (Lourens-Hattingh and Viljoen, 2001). Both bacterial genera produce inhibitory substances such as organic acids,

hydrogen peroxide, bacteriocins, and deconjugated bile salts in their metabolism (Lourens-Hattingh and Viljoen, 2001). Probiotics prevent and reduce tendency for diarrhea by competitive exclusion of pathogenic bacteria, protecting mucosal barrier layer, and improving immunity (Vasiljevic and Shah, 2008; Lourens-Hattingh and Viljoen, 2001). *Listeria monocytogenes*, *Salmonella* and *Helicobactor pylori* growth is inhibited by producing antimicrobial substances such as organic acids, bacteriocins, hydrogen peroxide etc. *Lactobacillus plantarum* and *Lactobacillus rhamnosus* exclude *Clostridium difficile* that causes antibiotic associated diarrhea. Probiotic *L. rhamnosus* GG, *B. lactis* Bb-12, *B. animalis* Bb-12 are good for shortening the duration of rotaviral infection in children. At the same time *L. rhamnosus* GG improves the rotavirus specific IgA production (Shah, 2007). Also there is evidence that some probiotic LAB reduces the severity of traveller's diarrhoea (Shah, 2007). The sysmptoms of irritable bowel syndrome have been improved when probiotics are used as a supplement with the standard therapy (Sanders and Marco, 2010).

Lactose intolerance. About 75% of the world population is lactose intolerant. The percentages can vary for different regions in the world (Granato *et al*, 2010). Lactose intolerance makes abdominal discomforts such as bloating, flateulance, nausea, abdominal pain, and diarrhoea to the host (Vasiljevic and Shah, 2008; Lourens-Hattingh and Viljoen, 2001). This discomfort is due to insufficient lactose digestion in the small intestine. The intensity of the discomfort depends on the load of consumed lactose and the length of transit (Vasiljevic and Shah, 2008). Studies have shown that fermented dairy products are better digested in lactose intolerant hosts (Vasiljevic and Shah, 2008).

Specially Bifidobacteria and yogurt bacteria with β-D-galactosidase hydrolyses lactose. This will increase the tolearance of lactose. But some LAB such as *L.acidophillus* does not hydrolyse lactose before consumption, but after consuming. Inside the gut these bacteria metabolize lactose improving the tolerance of the host (Lourens-Hattingh and Viljoen, 2001). By all means, part of lactose is consumed by the LAB in their metabolism and the load of lactose is lowered. There is a controversial situation regarding the improved lactose intolerance. That is the increased transit time of fermented dairy products due to high viscosity improves the digestion and absorption of lactose (Vasiljevic and Shah, 2008). The increased viscosity is a result of the exo-polysaccharide production by starter culture bacteria.

Antimutagenic and anticarsinogenic properties. A cancerous development is caused by the genotoxic effects of DNA. There are some clues that probiotics prevent or delay the occurrence of cancers. The postulated mechanisms are: exclusion of carcinogen/ mutagen producing bacteria in GI tract which lowers the carcinogen concentration, competition with bacteria that converts precarcinogens into carcinogens, and bioconversion of mutagens or direct inhibition of cancers by the bacterial metabolites (Saarela *et al*, 2000; FAO/WHO, 2001, Cenci *et al*, 2002). LAB and *Bifidobacteria* are responsible for decreasing the level of harmful bacterial enzymes such as β -glucuronidase, azoreductase, and nitroreductase which convert procarcinogens into carcinogens (Saarela *et al*, 2000; Vasiljevic and Shah, 2008). The organic acids, especially the butyric acid, contribute in lowering the levels and the effects of many mutagens such as 2-nitroflourene (NF) and aflatoxin-B. The suppressing effect was

higher in live cells than that of the dead cells (Vasiljevic and Shah, 2008). This emphasizes the importance of consuming live probiotics. Research was conducted by Cenci *et al* to monitor the effect of commonly used probiotic bacteria in commercial dairy substrates; *L. casei, L. plantarum, L. rhamnosus, L. acidophilus,L. delbrueckii and B. bifidum* against the genotoxic activity of 4-NQO (4-nitroquinoline-1-oxide – a genotoxin) in vitro (2002). The study proved that used cultures are effective on antigenotoxic activity against 4-NQO (Cenci *et al*, 2002). Long colonic transit is harmful as the length of contacting with putrefactive carcinogens is higher. By forming bulky stools, probiotic bacteria reduce the transit time for stools and the risk of colon cancers is reduced (Saikali *et al*, 2004). Nevertheless, the actual role of probiotics in cancer prevention is still to be disclosed (Saarela *et al*, 2000).

Increasing Mucosal Immunity. Probiotics have an influence on humoral, cellular, and innate immunity of the host (Gillingham and Lescheid, 2009). Adhesion of probiotic bacteria to the gut associated lymphoid tissue stimulates the immunity. LAB such as *L. johnsonii* LJ-1, *L. salivarius* UCC 118 stimulate IgA production and phagocytosis (Saarela, 2000). Probiotics promotes releasing cytokines from T helper (Th) cell sub groups (Th1 and Th2) (Gillingham and Lescheid, 2009). In healthy individuals, the Th1 and Th2 cell activity is balanced. However, the persistent production of Th 1, due to cytokine imbalance, leads to inflammation in gut mucosa. TNF – α is a pro-inflammatory cytokine, which is produced by the damaged gut mucosa. In such a case probiotics have the ability to balance the Th1 concentration by reducing TNF – α concentration (Gillingham and Lescheid, 2009). Although the mechanisms are not clearly

known, *Lactobacillus* species (*L. bulgaricus* and *L. casei*) inhibit the production of TNF by gut mucosa (Walker *et al*, 2006; Gillingham and Lescheid, 2009). The overproduction of Th2 cells are associated with allergies. The over expression of Th2 cell mediated cytokines is regulated by probiotics, such as *L. plantarum, L. lactis, L. casei and L. rhamnosus* GG (Gillingham and Lescheid, 2009).

Reducing Serum Cholesterol Level. High serum cholesterol is a risk factor for coronary heart diseases. Contribution of LAB on lowering the serum cholesterol has been monitored by using different animal models such as mice, pigs, and humans (Nguyen *et al*, 2007). In 1974 Mann and Spoerry observed hypocholesterol incident for the first time for a group of men, who were administered fermented milk. According to those studies, the suggested mechanism is the production of hydroxymethyl-glutarate by probiotics, which inhibits hydroxymethylglutaryl CoA reductase, a responsible enzyme in cholesterol synthesis (Vasiljevick and Shah, 2008). The other suggested mechanisms for hypocholesterol effect are absorbing cholesterol directly by cell membranes of probiotics, incorporating cholesterol for the growth, and precipitating cholesterol with bile acids resulting in the deconjugation of bile salts with fecal matter (Greany *et al*, 2008; Baroutkoub *et al*, 2010). A study done with *L. plantarum* PH04 (Dose is 4×10^8 CFU/ml) confirmed the safe use of this strain as a probiotic, and the cholesterol and triglyceride level of blood was significantly reduced. This was assumed due to decreased intestinal absorption of lipids or increased lipid catabolism (Nguyen *et al*, 2007). In 2010, Baroutkoub *et al* investigated consumption of probiotic yoghurt containing *L. acidophilus* and *Bifidobacteria* (dose is 10^6 CFU/ ml) by people in southern Iran reduced the level of

blood cholesterol. In contrast, another study in which subjects consumed capsules containing *L. acidophilus* strain DDS-1 and *Bifidobacterium longum* strain UABL-14 confirmed that the probiotics do not have a lowering effect of blood cholesterol (Greany *et al*, 2008). This latter study demonstrates that the food matrix plays a major role in reaching the gut as live cells by supporting the gut transit with acids and bile salts (Michida *et al*, 2006). The dose of probiotics required to exert the effect is not diagnosed (Ooi and Liong, 2010).

The effectiveness of probiotics

When selecting a probiotic for developing a probiotic containing food, the strain of the probiotic species to be used, the level of addition, toxicity, adaptation to the processing environment, stability during storage and changes in sensory properties should be counted (Champagne and Gardner, 2005). The biological properties, such as oxygen sensitivity, stability during storage, resistance to enzymes in the GI tract, sensitivity to lysozymes and phenolic compounds (due to amino acid hydrolyzation), antioxidative ability, and adhesion to animal cells should also be considered. All the properties cannot be seen in a single strain. Therefore using multiple strains would be beneficial (Champagne and Gardner, 2005).

In order to obtain the potential health benefit, the population of probiotics in a product, the viability of probiotic microorganisms and their ability to activate at the desired site in the alimentary canal are very important. The initial inoculum size of probiotics to selected food item is critical. The daily dose of probiotics is considered as 10^9-10^{11} CFU (Sanders, 1999). Hence consumption of 100 ml or g of a product bearing

the therapeutic minimum (10^6-10^8 CFU/ml or g of the product), would satisfy the daily requirement. The dose required for health benefits are variable. Due to lack of dose response studies, the dose required for each health benefit has not been identified. The population counts in the commercial functional foods also are needed to be identical to the claimed counts at the end of the shelf life. The shelf life is determined to maintain the desired level of probiotics even on the date of "best before" (Ooi and Liong, 2010).

The food matrix plays a major role in probiotic activity. Several factors are important for the growth and maintenance of viability of probiotics in food matrices. They are fat content, concentration and type of proteins, sugars or the concentration of carbohydrates and pH and oxygen level of the product (Champagne *et al*, 2005; Ranadheera *et al* 2010; Vinderola *et al*, 2011). Therefore, the product formulation plays an important role in the efficacy of probiotics and dairy products are ideal in this aspect. Consequently, dairy products are the best vehicles to carry probiotics in different forms, such as yogurt, cheese, butter milk, ice cream, and dairy desserts (Ranadheera *et al*, 2010). Due to high buffering capacity of cheese, experimentally it has been identified as a more efficient food matrix than yogurt to carry live probiotics to one's gut (Champagne et al, 2005). The food matrix itself and some added ingredients such as sweeteners, salts, aroma compounds and preservatives may affect the probiotic growth and their resistance to barriers inside the GI tract (Champagne *et al*, 2005; Vinderola *et al*, 2011).

Safety of Using Probiotics

LAB are renowned for safe use in food industry. Both *Lactobacillus* and Bifidobacteria are "Generally recognized as safe" (GRAS) due to several reasons. These two genera are abundantly consumed in fermented foods safely; these two genera naturally inhabit in the human GI tract (von Wright, 2005). Lack of successful isolation of these genera as primary pathogens and lack of side effects after consumption even by immunocompromised people lead to accept the probiotics are safe (Sanders, 1999). *Lactobacillus* and *Lactococcus* strains usually are considered GRAS, but some strains of *Strptococcus* and *Enterococcus* are opportunistic pathogens (Salminen *et al*, 1998). Specific guidelines are not introduced to determine the safety of administered probiotics. Therefore, the clinical safety of probiotic strains are tested by acute oral toxicity studies, colonization and translocation experiments with immunocompromised models, DNA based methods and degradation of intestinal mucus (Salminen *et al*, 1998; von Wright, 2005; Hempel *et al*, 2011). The infections associated with traditional probiotics are reported very rarely. The reported infections are gastrointestinal problems and those were fungemia due to *Saccharomyces boulardii* (Salminen *et al* 1998; Hempel *et al*, 2011). Though the traditional probiotics with a long history of safe use do not cause infections, the novel probiotic strains would not be the same.

Though there are not any recorded adverse effects, some enzymatic behaviors such as excessive bile salt deconjugation or degradation of mucus are assessed to study the intrinsic properties of a strain. The infective dose cannot be expressed as it is dependent on the microbial and host factors. The studies with animal models prove that

the LAB in functional foods does not cause any detrimental effects (Salminen *et al*, 1998).

Proper labeling is important for consumers to understand that the product is safe. In the USA, health related statements are not shown usually on labels. But in order to sell probiotic products, FDA approval or GRAS status is essential (Walker *et al*, 2006). The FDA strictly regulate labeling and marketing of probiotic containing functional foods (Brink *et al*, 2005).

The probiotics used in the USA are documented by the Food and Drug Administration (FDA). In South Africa Cosmetics and Disinfectants Act, 1972 (Act No. 54 of 1972) regulates the labeling and marketing of prebiotic and probiotic containing products (Brink *et al*, 2005). In Europe, probiotics for human consumption do not have accepted regulations, but some cases are monitored by EU Novel Food Regulation (258/97 EEC) (von Wright, 2005).

Some problems are associated with the safety evaluations due to lack of enough details of the administered probiotic organisms. The studies point out that there is not any increased risk of adverse effects when compared with control groups for strains of *Lactobacillus, Bifidobacterium, Saccharomyces, Streptococcus, Enterococcus,* and *Bacillus*. Some adverse effects were observed for some case studies after administration of probiotics (within 3 days to several weeks), but these kinds of things are not systemically reported (Hempel *et al*, 2011).

Most studies have been done for *Lactobacillus* interventions, and a few on *Saccharomyces* and *Bifidobacterium.* Though there are some records for hospitalizations

after consuming foods containing *Saccharomyces, Lactobacillus,* or *Bacillus* strains, there is not evidence to prove those effects were due to probiotics (Hempel *et al*, 2011). *Enterococci* has been shown to act as an opportunistic pathogenic behavior in nosocomial environments causing endocarditis, bacteremia and intra-abdominal, urinary tract and central nervous system infections (von Wright, 2005)

The ability to carry antibiotic resistant genes by probiotic bacteria is also important in considering the safety of probiotics. LAB also carries and receives antiobiotic resistant genes through bacterial conjugation, but this resistance is intransmissible (von Wright, 2005). Some *Lactobacilli* strains show vancomycine resistance.

Probiotics Needs Foods--Prebiotics

Prebiotics play an important role enhancing the growth of probiotics. Prebiotics were first defined by Gibson and Roberfroid in 1995 as "non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth and/or activity of one or a limited number of microbes in the colon resulting in documented health benefits" (Forssten *et al*, 2011; de Vriese and Marteau, 2007; Bosscher *et al*, 2006; Gibson and Roberfroid, 1995). A compound should have several characteristics to be a prebiotic. It should not be hydrolyzed or absorbed in the upper part of the GI tract; the compound should be fermented selectively by intestinal flora; it should induce the growth of selected number of beneficial bacteria (Bosscher *et al*, 2006).

The undigested fibers that enter the large intestine are metabolized by the endogenous microorganisms. Fructo-oligosaccharides (FOS), inulin,

galactooligosaccharides (GOS), isomolto–oligosaccharides (IMO), polydextrose, lactulose, lactilol and resistant starch are the main types of prebiotics. (Siro *et al*, 2008; Forssten *et al*, 2011). Though most prebiotics are commonly considered as "dietary fibers", all dietary fibers do not possess prebiotic properties (Forssten *et al*, 2011). Some authors suggest only inulin and inulin type fructans and GOS satisfy the criteria to be prebiotics (de Vriese and Marteau, 2007; Gibson and Roberfroid, 1995). Most prebiotics, such as FOS, GOS and inulin have been documented to increase the level of fecal bifidobacteria (Forssten *et al*, 2011). *Bifidobacteria* is advantageous in consuming FOSs from inulin due to possession of β-1, 2-glycosidase enzyme (de Weile *et al*, 2004). Therefore, the purpose of taking prebiotics is to increase the population of 'friendly bacteria' and improve the resistance to pathogenic bacteria (Forssten *et al*, 2011).

The world demand for prebiotics is 167,000 tons approximately (Siro *et al*, 2008). Consumption of prebiotics changes the composition and metabolism of intestinal microbiota (Forssten *et al*, 2011; de Vriese and Marteau, 2007). The metabolism increases the bacteria biomass and the levels of short chain fatty acids (SCFAs), such as acetic, propionic and butyric acid. These three fatty acids fulfill body's energy requirements at different sites. Acetic acid is an energy source in muscle cells. Propionic acid forms ATP in the liver and butyrate is the energy source for colonocytes. The high bacterial biomass makes the stools bulky (Bosscher *et al*, 2006). SCFAs lower the pH of the surrounding suppressing the growth of pathogenic bacteria (Forssten *et al*, 2011, de Vriese and Marteau, 2007). These weak acids enter the cells passively in protonated form and dissociates inside the cells lowering pH of the cytoplasm. The accumulating H^+ ions

due to dissociation of weak acids make a trans- membrane proton gradient. The result is proton motive force, which utilizes ATPs of the cell. Additionally, internal high acidity may affect the enzyme inactivation and damage DNA and other proteins (de Guchte *et al*, 2002). These compounds not only play a role as prebiotics, but also accelerate the Ca, Mg and Fe absorption and hence improve the bone mineral content and bone mineral density in humans and rats (Siro *et al*, 2008; de Wiele *et al*, 2004). Short-chain fatty acids results in digestion of prebiotics serve as energy sources too. Butyric acid is the main energy source of colonocytes. Acetic acid and propionic acid enter the systemic blood circulation and enter the liver. Acetic acid is metabolized in muscle tissues too and propionic acid is metabolized through gluconeogenesis too (Forssten *et al*, 2011; Bosscher *et al*, 2006). Energy value estimated for inulin is 4.2 kJ/ g and 6.3 kJ/ g for oligofructose (Bosscher *et al*, 2006).

Fermentation time for prebiotics depends on the degrees of polymerization (DP). For inulin, the fermentation time could vary from 5-15 h according to the DP. Prebiotics with a low DP are usually fermented in proximal end of the colon and prebiotics that slowly ferment reach the distal end (Bosscher *et al*, 2006)

Formulation of a probiotic product with prebiotics gives symbiotic properties to the food item. Added prebiotics supports the growth and survival of exogenously administered *Bifidobacteria* and *Lactobacilli* inside the gut (Takemura *et al*, 2010). *In vitro* studies show that inulin and oligofructose with *L.plantarum* and *B.bifidum* increase the bifidogenic activity and decrease the pathogens, such as *Campylobacter jejuni*, *E. coli*, and *Salmonella enteritidis* (Fooks and Gibson, 2002). Also, administering 2g/day of

oligofructose for 4 weeks to 6-24 months infants has increased the *Bifidobacteria* growth and decreased the *Clostridium* growth (de Vriese and Marteau, 2007). Additionally, the studies by de Wiele points out that it is not to take a single dose of inulin, which is the most effective prebiotic. A significant growth of populations of *Bifidobacteria* and *Lactobacilli* were observed only after a three week of supplementation of inulin. The dose was 2.5g/day. In order to maintain a better level of probiotic bacteria in the colon, inulin should be taken continuously (de Wiele *et al*, 2004).

The recommended daily dose of prebiotics is 4g (Forssten *et al*, 2011). The recommended daily dose of inulin is 1-4g (de Wiele *et al*, 2004). Some authors mention the dose for inulin-type prebiotics as 2.5 – 5g/ day in order to have a bifidogenic effect (Kelly, 2008). The overconsumption may cause diarrhea. There is not enough evidence to conclude the clinical importance of prebiotics for the reduction of diarrhea for humans (de Vriese and Marteau, 2007). Several studies revealed that the consumption of chicory root inulin lowers the triglyceride level in blood and consequently a hypo cholesterol effect was observed (Ooi and Liong, 2010).

Non-Dairy Probiotics

It is well documented that dairy products serve as the best substrate for probiotic bacteria. However, a trend of non-dairy probiotics is growing due to some issues in dairy probiotics. The dairy substrates may contain potential allergens, such as casein and cold storage during the shelf life is required. The cholesterol content of dairy products is high. Therefore, the demand for new tastes with non-dairy matrices and the trend of vegetarianism are increasing (Ranadheera *et al*, 2010). Also producing probiotic products

with foods and beverages which are part of the day-to-day life is encouraged. This leads to increased demand for non-dairy probiotic foods, such as coconut milk, fruit drinks, nutrition bars, soy products and cereal-based products. The nutritive values and large distribution of these new raw materials are important in using these new substrates for functional food items (Angelov *et al*, 2006). In the development of novel probiotic products fermentability of the substrate by starter culture and increasing the viable cell concentration should be considered. The expectation is to have a healthy product without lactose intolerance and free of allergenic milk protein. Most vegetarians prefer milk free probiotics (Granato *et al*, 2010; Rivera-Espinoza and Gallardo-Navarro, 2010).

Cereal products. Cereal production comprise approximately 60% of the total world food production (Salmeron *et al*, 2009). Cereals are inexpensive substrates that are commonly used in LAB fermentation. Cereals are rich in proteins, carbohydrates, vitamins, minerals and non-digestible components fulfilling too many nutritional requirements of probiotic bacteria (Rivera-Espinoza and Gallardo-Navarro, 2010). Therefore, using those substrates for fermentation is very appropriate industrially. The first non-dairy probiotic product PROVIVA is fermented oat gruel with *Lactobacillus plantarum* 299v (Prado *et al*, 2008).

Cereals, having prebiotic properties, are becoming popular substrates for probiotic bacteria (Charalampopoulos *et al*, 2002). Cereal substrates are not only inexpensive but also renewable. Fermented cereal products are produced and consumed by individual and mixed cereals. Boza (fermented wheat, rye, millet, maize and other cereals), bushera (sorgum or millet flour), mahewu (corn meal with sorgum), pozol (cooked maize) and

togwa (maize flour and finger millet malt) are some traditional fermented cereal products in different regions of the world (Prado *et al*, 2008). Several experiments have been set to test the efficacy of oat as a vehicle for probiotic bacteria.

In 2006, Angelov *et al* successfully prepared an oat based drink using *L. plantarum* B28 using whole grain oat as the substrate. Martensson *et al* produced another successful oat based non-dairy probiotic product in. Oatmeal, a common hot breakfast option, is a promising cereal matrix for most probiotics. The cereals with bran are a rich source of proteins due to aleuronic layer, fibers and minerals such as calcium, potassium, sodium and magnesium (Kedia *et al*, 2008). Beta-glucan in oat bran, is a kind of beta gluco-oligosaccharide, is a prebiotic that stimulates the growth of *Bifidobacteria* in gut (Angelov *et al*, 2006). However, studies show oats are not as efficient as dairy or soy matrices (Sanders and Marco, 2010). Wheat, malt and barley have also exhibited the suitability of those cereals as carriers for probiotics (*L. fermentum, L. reuteri, L. plantarum* and *L. acidophilus*) maintaining counts > 10^7 CFU/ml after fermentation at 37 ^{0}C for 48 hours (Charalampopoulos, 2002).

Beverages. Soy milk and fruit- and vegetable-based substrates are used in manufacturing fermented beverages. Fermented coconut water is also available in supermarkets as a functional drink. Coconut (*Cocos nucifera*) is a perennial plant that grows in tropical regions. Coconut water is the liquid endosperm of the coconut fruit. Young coconut or green coconut, which is harvested at 6-7 months of development, is a sterile, nutritious and refreshing drink (Yong *et al*, 2009; Campos *et al*, 1996). The main component of coconut water is water (*ca.* 94 %,). There are 5-6 % of total sugars

(sucrose, glucose, and fructose) and 19.5 % of protein. Other components found in coconut water are sugar alcohols, lipids, amino acids, nitrogenous compounds, organic acids, enzymes, vitamins, and micronutrients.

Fruit juices are also fermented with LAB strains, which can tolerate a high acidic pH. Usually the pH of fruit juices is between 2.5 to 3.7. Therefore, this is a microbiologically safe matrix (Sheehan *et al*, 2007). High acidity (> pH 4.0) may lead to a poor viability. This can be compensated by mixing fruit pulps with milk (Champagne and Gardner, 2008). However, when fruit juices are fermented with probiotic bacteria, unfavorable aroma and flavors can be added. This may cause consumer disapproval (Rivera-Espinoza and Gallardo-Navarro, 2010). In a study of assessing the viability in pineapple juice and orange juice, *L. rhamnosus*, *L. casei* and *L. paracasei* showed a good viability (over 10^7 CFU/ml) for 12 week period under refrigeration temperature (Sheehan *et al*, 2007). Therefore some fruit juices are promising substrates for some strains of probiotics under proper storage conditions. Vegetable juices, such as carrot juice, tomato juice, beetroot juice, cabbage juice, cucumber juice and garlic, have been tested experimentally by LAB (Granato *et al*, 2010; Rivera-Espinoza and Gallardo-Navarro, 2010). Fermented tomato juice (for 72h) by *L. plantarum*, *Latobacillus acidophilus, Lactobacillus casei and Lactobacillus delbrueckii* have survived for four weeks in the refrigeration temperature (4 ºC) with over 10^8/ml counts (Yoon *et al*, 2004).

Probiotic Chocolates. Chocolate has also been tested as a new probiotic carrier to the GI tract (Possemiers, 2010). The studies have obtained the counts as over 8 log10 CFU/g for microencapsulated LAB and *Bifidobacteria*. Studies by Possemiers

(2010) have used a simulator of human GI tract (SHIME) to model the viability inside the GI tract. The observation is viability of chocolate is 5-fold higher than the viability of milk inoculated with the same probiotics and inoculum size.

Rheology of Probiotic Food Matrices

Polysaccharides play an important role in the food industry as emulsifiers, stabilizers, gelling agents, thickeners and are also used for encapsulation. Microbial exposolysaccharides (EPS) are getting attention as a natural thickener. Most of the economically important bacterial EPS are produced by LAB, which are manipulated as probiotics to improve rheology and texture of fermented dairy products. More efficient polymers are synthesized chemically to improve the above functions, but consumption of the synthesized polysaccharides is not accepted in all countries. In European countries, EPS producing starter cultures are available for yogurt production, as using stabilizers is banned (de Vuyst and Degeest, 1999). Approximately 30 species of LAB produce EPS. The most popular probiotic dairy product yogurt possesses the characteristic texture and increased viscosity due to presence of EPS. The production of EPS does not add any flavors to the product (Badel *et al*, 2011). EPS are high molecular weight carbohydrates with a repetition of monosaccharide monomers; D-galactose, D-glucose and L-rhamnose (Khurana and Kanawjia, 2007). EPS production is dependent on the nutrients available in the medium (sugars, amino acids and vitamins) and other factors that support the growth of bacteria such as temperature, pH, oxygen tension, and incubation time (de Vuyst and Degeest, 1999). The production of EPS by mesophillic LAB such as *L. planatarum* is not

growth associated, EPS production in thermophillic LAB, such as *S. thermophillus* is growth associated (De Vuyst *et al* 1998; De vuyst and Degeest, 1999).

Rheology is the science of deformation and flow of a material. Rheology of a food matrix is very important in several ways such as process engineering, determination of the ingredient functionality in product development, quality control, shelf-life analysis and food texture which is related to sensory analysis of the food item (Steffe, 1996). The increased viscosity lengthens the gut transit allowing an efficient digestion of lactose in fermented dairy products (Vasiljevic and Shah, 2008).

The two basic concepts of rheology are stress and strain. According to Hooke's law stress and strain are proportionally related and the propertionality constant is named as "viscosity", which is the internal resistance of a fluid to flow (Nielsen, 2003). For Newtonian fluids, the viscosity is constant for all shear rates. Water, raw milk, olive oil, air, cotton seed oil, and honey are some examples for Newtonian fluids. Thus for Newtonian fluids, the graph of shear stress (τ, Pa) against shear rate (γ, s^{-1}) is a straight line which goes through the origin. Many food items are non newtonian fluids which do not show a constant viscosity when shear rate is changed. Ketchup, mayonnaise, and salad dressings are some non-Newtonian fluids. Non newtonian fliuds have an apparent viscosity, which is dependent on shear. If the viscosity of a product decreases as shear rate increases, the fluid is described as shear thinning or pseudoplastic. If the viscosity increases with the shear rate, the fluid is shear thickening or dilatent. Shear thinning and thickening effects are time independent.

Many non-Newtonian fluids have a yield stress, which is the minimum force (stress, τ_0) required for inititaion of flow. Both pseudoplastic and dilatent fluids follow the Ostwald de Waele power law model, where the yield stress equals to zero and can be expressed as, $\tau = K \gamma^n$, where, τ is the shear stress (Pa), γ is the shear rate (s^{-1}), K is the consistancy coefficient (Pa s^n) and n is the flow behavior index of the material, which is a dimentionless parameter and indicates how close the liquid is, to Newtonian flow. For pseudoplastic fluids $n<1$ while $n>1$ for dilatent fluids.

Viability of Probiotics

The probiotic potential of a product reflects the survival of the probiotics in the GI tract. Survival of probiotics at different levels of GI tract, is highly dependent on several factors including the strain characteristics, nutrient composition, oxygen content, pH, water activity, presence of other bacteria, storage time and temperature (Salminen *et al*, 1998; Granato *et al*, 2010; Champagne, 2009; Sanders and Marco, 2010). Intra-individual conditions of consumers are also significant in this, but it cannot be measured. The food serves as the nutrient source for both consumer and probiotic bacteria, and that is the carrier of probiotics to the gastrointestinal tract. The growth of probiotics in food matrices is better in the presence of bioactive ingredients such as prebiotics,vitamins, minerals, fibers, enzymes, food preservatives and flavors (Sanders and Marco, 2010). Several factors contribute for the survival and activity of probiotics in a food item. They are, the physiological state of the added microorganism (the stage in the growth cycle), the concentration of viable cells by the time of consumption, physical conditions during storage such as temperature fluctuations, the chemical composition of the product and

some interactions between probiotics and starter culture. (Rivera-Espinosa and Gallardo-Navarro, 2010).

Having a fermentative metabolism, probiotics make the food matrices acidic and high acidity is detrimental for their survival. Fruit juices are therefore mixed with dairy ingredients to introduce a better nutritional matrix with a higher pH. Studies on probiotics point out that the probiotic bacteria gain resistance to pH stress during the shelf life. The probiotics with induced resistance for acidity can tolerate heat, oxygen and osmotic stresses better than a fresh culture. The same study reveals cultures with the poorest stability have the highest viability loss in gastric acid environment with some *in vitro* studies (Champagne and Gardner, 2008). Though the terms for shelf life of a probiotic product have not been clearly defined, taking the viable count above 10^6 CFU/ml and pH above 4.0 is consireded as reasonable in determining the shelf life (Angelov *et al,* 2006).

Lactic Acid Bacteria

LAB are a group of bacteria that consists of several genera and numerous species. Usually, these are Gram positive, non-motile, non-endospore forming rods, coccobacilli, or cocci-shaped bacteria. LAB are catalase negative and grow under microaerophilic conditions (Holzapfel and Stiles, 1997; Bonaparte *et al,* 1998). The genus *Lactobacillus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, family *Lactobacillaceae*.

When classifying the LAB into genera, the carbohydrate fermentation patterns, NaCl tolerance ability, growth on different media, growth at specific temperatures and antibiotic resistance are useful (Bonaparte *et al,* 1998).

LAB are also chemaorganotrophic (Bonaparte *et al*, 1998). According to the pattern of hexose metabolism, LAB are divided into two groups: heterofermentative LAB and homofermentative LAB (Corsetti *et al*, 2005; Ali, 2010). In homofermentation, single glucose molecule is converted to two lactate molecules by Embden–Meyerhof–Parnas (EMP) pathway (Figure 1) (Bustos *et al*, 2005). In heterofermentation, sugar degradation occurs via phosphoketolase (PK) pathway (Figure 2). "Lactobacillus" group has three divisions based on carbohydrate metabolism. They are obligate homofermenters (*L. acidophilus*, *L. delbruckii*), facultative heterofermenters (*L.paracasei*, *L. plantarum*, *L. pentosus*), and obligate heterfermentors (*L.brevis* and *L. fermentum*).

***Lactobacillus plantarum* as a probiotic bacterium**

Lactobacillus plantarum, a member of the LAB group, is a Gram positive, rod shaped, non-endospore forming bacterium. Cells may appear as single cell, in pairs, or sometimes arranged in a line. The fermentation pattern is homofermentative, resulting in lactic acid as the sole end product of carbohydrate fermentation of this mesophilic microorganism. *L. plantarum* is confirmed as homofermentative due to lack of production of acetic acid and ethanol (Giraud *et al*, 1991; Charalampopoulos *et al*, 2002). However, sometimes it is described as heterofermetative lactobacilli. The fermentation pattern is determined by the composition of the food matrix. *L. plantarum* become heterofermentative when the medium contains more glucose and under aerobic conditions converting pyruvate to acetate by an oxygen-dependent pyruvate oxidase and H_2O_2 is formed in the reaction (Murphy and Condon, 1984).

Lactobacillus plantarum is naturally found on plant surfaces, and therefore, this is a common microorganism in fermented plant products, such as brined olive and sauerkraut and also on fermented dairy and meat products. This bacterium, also found inside the healthy human GI tract, is a prerequisite to becoming a probiotic bacterium (de Vries *et al*, 2006; Ahrne´ *et al*, 1998). Genome analysis confirms that *Lactobacillus plantarum* can use various sugars, and large numbers of genes that encode the regulatory functions confirm that it can acclimatize to different environments (de Vries *et al*, 2006). Several strains of *Lactobacillus plantarum* are marketed in probiotic industry. *Lactobacillus plantarum* 299v and *Lactobacillus plantarum* Lp 115 are commonly used commercial starter cultures.

Lactobacillus plantarum has a long history of safe use for olive and sauerkraut fermentation. A study of intravenous injection of rats with 10^8 CFU *Lactobacillus plantarum* 299v, showed there were not any bacteria isolated from heart and blood. Several clinical studies have proven the safe use of *Lactobacillus plantarum* without abnormal translocation (de Vries *et al*, 2006). Most *L. plantarum* spp are able to survive in the GI tract for at least one week after administration.

Lactobacillus plantarum isolated from food samples and from human GI tract have a very high tolerance of low pH and bile salts (de Vries *et al*, 2006). When *L. plantarum* Lp-115 was tested for the resistance to pepcin, pancreatin, and bile, significantly high survival rate was observed (Daniel *et al*, 2006). In a study of some *L. plantarum* strains isolated from cheese showed preservatives frequently used in dairy industry, such as calcium propionate do not affect the growth and *L. plantarum* is able to

grow when the NaCl concentration is 0.1-4.2 %. The *L. plantarum* isolated from sausages grew in a high salt concentration of 6.5-10 % (w/v) (Georgieva *et al*, 2009).

L. plantarum Lp 115-400B can survive in the mice gut even after stopping the continuous feeding of the probiotic strain. After mice were intragastrically injected a mixture of 10^{10} CFU LAB for four days, *L. plantarum* Lp 115-400B was recovered after 13 days in fecal matter (Daniel *et al*, 2006). The viable counts in feces of *L. plantarum* is too low to detect if it was not orally consumed. The detected amounts were low as 3.2 x 10^4 CFU/g (de Vries *et al*, 2006).

Lactobacillus plantarum has the ability to adhere to the mannosylated cell-bound receptors in gut. Gram negative pathogenic bacteria such as *E. coli* also compete for these receptors, but in the presence of *Lactobacillus plantarum*, pathogenic adherence is retarded (de Vries *et al*, 2006). *Lactobacillus plantarum* has a good ability of co-aggregation, which facilitates the colonization of probiotics in the GI tract (Collado *et al*, 2008).

Most LAB cannot hydrolyze starches. Some isolates of *Lactobacillus plantarum* strains such as *Lactobacillus plantarum* A6 and *Lactobacillus plantarum* D34 possess the amylase activity (Prado *et al*, 2008). Other than cereals *L. plantarum* spp. is able to successfully ferment fruit juices and vegetable juices (tomato, beetroot) with a high viability (Santo *et al*, 2011)

Lactobacillus plantarum is also used with prebiotics to yield symbiotic products. According to published studies, it can ferment FOS, GOS and lactulose, but not inulin (Georgieva *et al*, 2009). 1998 Kontula *et al* showed that the xylo–oligosaccharides

available in oats can be used only by *Lactobacillus plantarum*. In an experiment to test the ability to ferment oat bran using LAB, the best cell concentration resulted when *Lactobacillus plantarum* was used.

Lactobacillus plantarum is productive against many diseases. The diarrhea associated with *Clostridium difficile* showed a less opportunity of recurrence when antibiotics were administered with a *Lactobacillus plantarum* strain (de Vries *et al*, 2006). The symptoms of irritable bowel syndrome were less intensive when *Lactobacillus plantarum* was consumed (de Vries *et al*, 2006).

The probiotic potentials of different strains are variable. It is better to use several strains of *Lactobacillus plantarum* with different potentials (Cebeci and Gurakan, 2003). Under the environmental stresses, *Lactobacillus plantarum* become viable but non culturable state (Oliver, 2005).

Testing Viability of Probiotics

Good viability is a prerequisite for the functionality of probiotics. Viability is considered as a measurement of the probiotic activity (Charalampopoulos *et al*, 2002). Viability of the probiotics would affect the prevailing gut flora of the host quantitatively and qualitatively (Fuller, 2006). The term viability is controversial without a proper definition. Earlier viability was considered as the ability to grow *in vitro* (Kell *et al*, 1998).

Viability of microorganisms can be tested in several ways. The simplest and the most traditional way is to culture a suitable dilution on an agar and count the colonies after incubation. Here, a count is taken for visible colonies on the agar after the set

incubation conditions or culturability is monitored. Another method of enumerating the viability is taking most probable number (MPN) (Kell *et al*, 1998). Not only the culturable cells, but also dormant, non-culturable cells are available in a food item and these cells will not produce colonies on an agar. Cells become non-culturable due to numerous reasons. Mainly this state is induced due to natural stresses such as lack of nutrients or elevated nutrient levels in culture media, incubation outside the desired temperature range, salinity changes, oxygen concentration and exposure to white light (Oliver, 2005; Kell *et al*, 1998). Any sub-lethal injury will prevent the dividing of cells such as damages to DNA, transcription factors or ribosomes. The minimum amount of these components for regrowth is not known (Kell *et al*, 1998). Stress responses are variable of the species and available stresses. When the non culturable cells are recovered, those have the ability to grow again. These non culturable cells are metabolically active and live (Diaz *et al*, 2010, Kell *et al*, 1998). Therefore the physiological state of a cell cannot be confirmed as "live or dead" considering the culturable or non culturable nature and taking the number of colonies as the viable count underestimates the "viability" (Kell *et al*, 1998).

Enumeration Methods of Viability of Probiotics

Plate counting is the most traditional standard method of counting viability. It actually counts the number of bacteria that can replicate and appear as colonies under the handled conditions (Kell *et al*, 1998; Amor *et al*, 2002). Lactic acid bacteria are cultured on MRS agar (de Man Rogosa and Sharpe agar) and *Bifidobacteria* is usually cultured on Reinforced Clostridial (RC) agar. MRS agar supplies required carbon and nitrogen

sources for bacteria and other growth factors are supplied by magnesium and polysorbate 80. Acetate, citrate and manganese inhibit the growth of other bacteria in the sample. RC agar medium supplies nutritional requirements and cystein hydrochloride is a reducing agent, which is suitable for anaerobes. The inoculated plates are anaerobically incubated at 37 ºC for 48 to 72 hrs (Lahtinen *et al*, 2006). The disadvantage of this method is not getting reproducible counts as the count depends on the medium, incubation pattern selected and on the influence of neighbor cells. Sometimes the colonies would be due to a clump of cells, though it is considered as a single cell (Kramer, 2009). Consequently, the true count may be underestimated.

Molecular methods are also used to ensure the viability of cells. The half-life of m- RNA is too short and, therefore, it is a good indication of viability of cells. The m-RNA can be detected using reverse transcriptase polymerase chain reaction (RT-PCR) and nucleic acid sequence based amplification (NASBA) (Keer and Birch, 2003). One advantage of these methods is that they provide more reliable results than conventional culturing methods as some cells may be dormant losing the ability to produce colonies on agar media. As m-RNA also has a very short half-life, usually many genetic identification procedures are based on detecting 16s r RNA. Having a shorter half-life than DNA, this is assumed to be a better option for viability testing. 16s r RNA is mostly assayed using polymerase chain reaction. Fluorescence In Situ Hybridization (FISH) is another viability testing method for 16s rRNA assay. The r RNA is coupled with a fluorescent labeled oligonucleotide probe, and the cells are observed under fluorescent microscopy or flowcytometry. The presence of r RNA depends on r RNA decay after cell death. Real

time PCR is another cell quantification method where DNA is used. The favorable target DNA is amplified using species specific fluorescent labeled primers in the presence of quencher primers. The intensity of fluorescence is measured, which is proportional to the number of target DNA copies. When the above two methods are compared, almost identical results obtained for a study done with fermented oatmeal by *Bifidobacteria* (Lahtinen *et al*, 2006). Therefore, both of these methods are useful in the case of determining the total cell count.

Flowcytometry (FCM) analysis, which is used for many environmental samples, is a rapid method of counting cells. Flowcytometry was a familiar technique in research since 1975 for the analysis of bacteria and yeast (Laplace-Builhe *et al*, 1993). This measures a very large number of cells within a very short period of time. Usually this value is 5000 cells per second. Flowcytometry is a destructive method of sample analysis. A flowcytometer consists with several units such as the light source, flow cell and hydraulic fluidic system, optical filters, photodiodes and data processing unit (Diaz *et al*, 2010). Data is acquired graphically as dot plots, which are named as cytograms. The dot pattern helps to differentiate different populations according to the physiological state of cells, gates are fixed to select the interested population.

Flowcytometry accompanied with fluorescent staining methods are also useful in viability determination. The intergrity of cell membranes is frequently analyzed in viability assesments by fluorescent staining. The cells with damaged memebranes are unable to maintain the membrane potential and therefore these cells are considered as dead cells. The membrne intergrity is detected by fluorescent dyes (Diaz *et al*, 2010).

The dye enters the cell through the membrane and the DNA of cells is stained. Live/dead BacLight™ assay, which is an affordable and relatively rapid method, is often helpful in viability counting studies. Live/dead BacLight™ is a combination of two fluorescent dyes which are made to stain the cells according to cell membrane integrity. Membrane integrity is symbol of the viability of cells. SYTO® 9 and PI are the fluorescent staining agents in this kit (Diaz *et al*, 2010). SYTO® 9 is a membrane permeant nucleic acid stain that stains both live and dead bacteria in green and propidium iodide (PI) is a red fluorescent dye that stains bacteria with damaged membranes (AlakomI *et al*, 2005). Some incidences have been reported that PI stained the DNA of intact cells and cells of log phase. Heat treated dead cell can be used as the control and thick sample should be homogenized gently to release bacteria trapped in matrix. Comparing the live/ dead cell count and plate count states the culturable and non-culturable cells in the sample (Lahtinen *et al*, 2006). The ability of the dormant cells (non-culturable cells) to act as functional probiotic cells is yet to be discovered. Live/dead cell counts are evidence that there are non - culturable cells with the integrated cell membrane (Lahtinen *et al*, 2006). Not only the enumeration, but also the identification is important in these applications as there is evidence that probiotic products are mislabeled (Coeuret *et al*, 2004). This would be a potentially reliable method in monitoring the microbial dynamics in a fermentation process (Malacrino *et al*, 2001).

Bacterial Growth and the Influence of Temperature.

Bacterial growth at a constant temperature in a suitable medium has different phases. They are lag, log, stationary and death phases. The phases are named according to

the growth rate at each phase for a selected strain of microorganism. The growth of bacteria can be expressed as 'exponential' at the log phase and mathematically this is denoted as,

$$N = N_o \exp^{kt} \qquad (1)$$

N is the number of cells after t time, N_o is the initial number of bacteria, k is the specific growth rate and t it the time taken. As we are interested on the growth rate, the equation can be re-arranged as,

$$\ln N = \ln N_o + kt \qquad (2)$$

The Arrhenius equation approach was used to describe the changes of specific growth rate (k) of living microorganisms according to temperature changes. Mathematical expression of Arrhenius equation for chemical reaction is,

$$k = A \exp^{-Ea/RT} \qquad (3)$$

$$\ln k = \ln A - (Ea/R)\,(1/T) \qquad (4)$$

In equation 4, Ea/R is the slope, Ea is Arrhenius activation energy for the growth, A is the frequency factor, which is independent of temperature and with units of k, Ea is the activation energy, which is the minimum energy required for the occurrence of reaction, T is the absolute temperature (in Kelvin) and *R* is the universal gas constant 8.31J/mol K.

According to the Arrhenius equation, chemical reaction rate increases with increased temperature. However for biological systems this is different. *Lactobacillus plantarum* Lp 115-400B, which is a mesophillic bacterium, whose optimum temperature is between 27-30 °C increased temperatures may retard the growth instead of enhancing it.

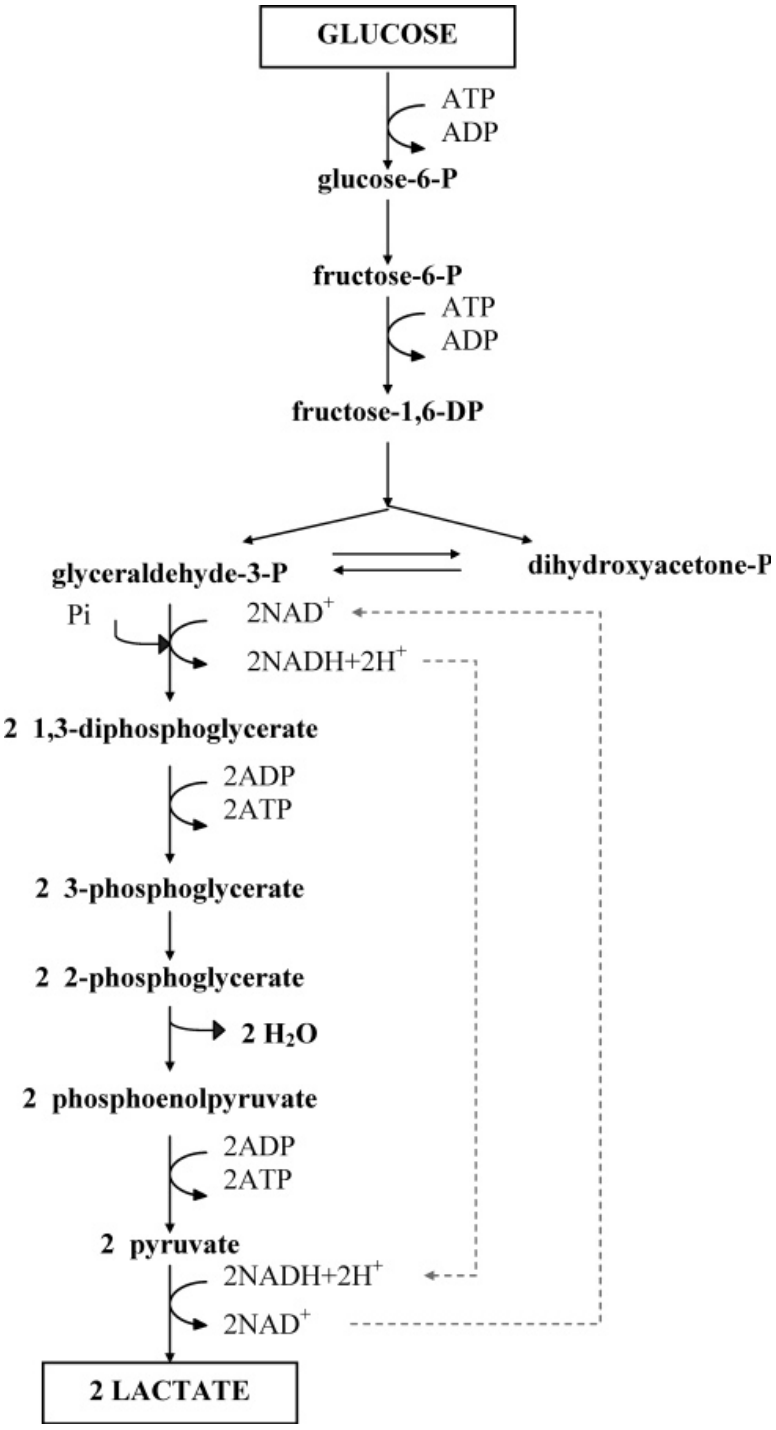


Figure 1: Homofermentative Fermentation of LAB (Bustos *et al*, 2005).

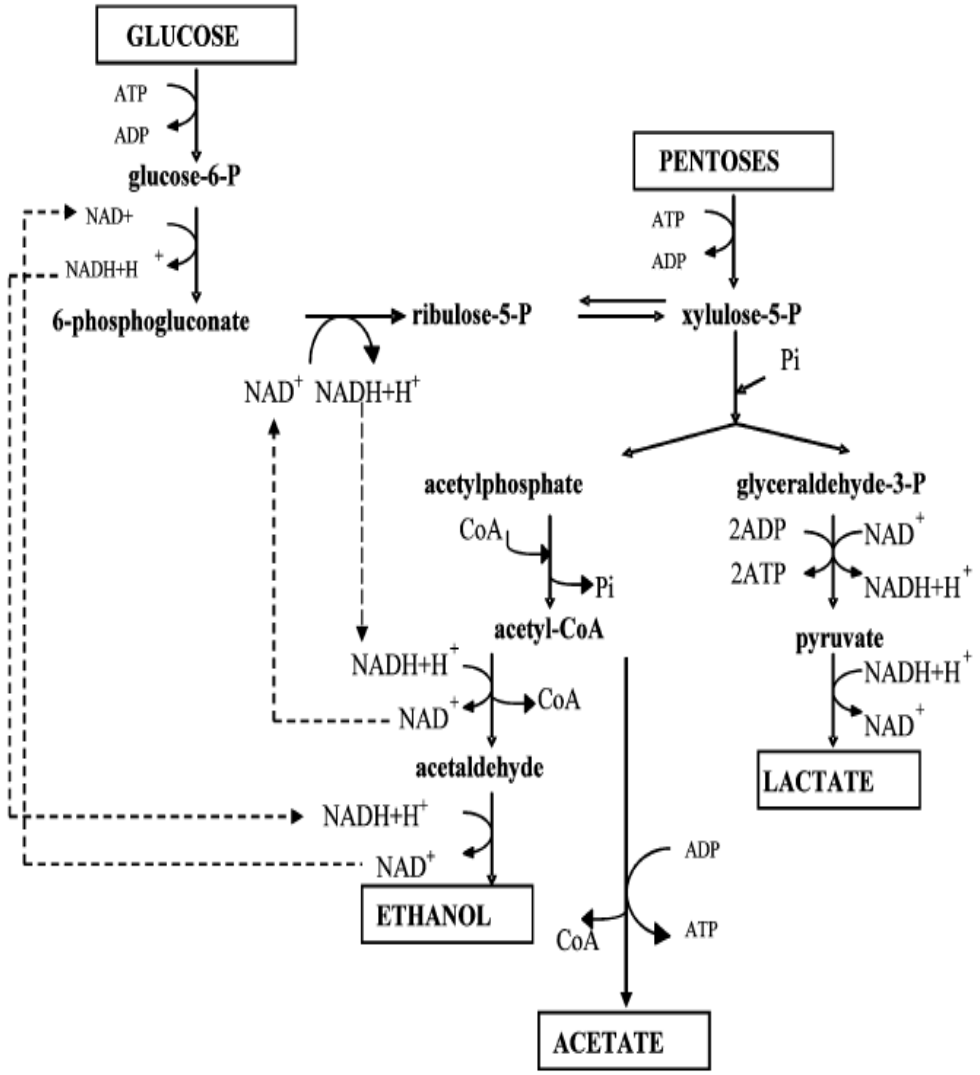


Figure 2: Heterofermentative Fermentation of LAB (Bustos *et al*, 2005).

MATERIALS AND METHODS

Study I

Commercial Probiotic Products.

All probiotic food products tested were bought from a local supermarket. The products tested are shown in Table 1. Products were refrigerated immediately after purchasing as recommended by manufacturers for all products. The samples were kept under refrigeration temperature (4±1 ºC) throughout the entire shelf life assuming the temperature represents a commercial cooler. The temperature of the refrigerator was monitored during the storage time.

Products FCW and FCWI were fermented coconut water of two brands. FCWI is a symbiotic product as the product contains inulin as a prebiotic. RKWF is a raw product as it is a naturally fermented unpasteurized beverage which contains organic kombucha, raspberry juice, lemon juice, and ginger juice. FJPB is a probiotic fruit juice enriched with multi vitamins, which is available in different flavors. For the experiment, the pomegranate blackberry flavor was used. FDD is a fruit flavored fermented dairy product. MCB is a probiotic chocolate bar; a solid matrix with a low water activity.

Table 1: The commercial probiotic products assessed for the viable count analysis

Product	**Physical status of the product**	**Number of samples (n)**	**Claimed probiotic species**	**Claim by the manufacturer on the label**	**Capacity of the container**	**Serving size**
Fermented coconut water - FCW	Liquid	3	*L.plantarum* 299v	2×10^{10} cells /container	960 ml	240 ml
Fermented coconut water with inulin - FCWI	Liquid	2	*L.plantarum, L. paracesei*	2×10^{10}cells /container	480 ml	240 ml
Raw Kombucha with raspberry juice, lemon juice and ginger juice -RKWF	Liquid	3	*Lactobacillus* bacterium, *Saccharomyces boulardii*	1×10^{9}cells from each probiotic/ serving size	480 ml	240 ml
Fruit juice with pomegranate blackberry flavor - FJPB	Liquid	3	*L. plantarum* 299 v *Bifidobacterium lactis*07	Not declared	320 ml	80 ml
Fruity dairy dessert - FDD	Liquid	5	*L. acidophilus, L casei*	5×10^{10}cells /container	105 ml	105 or 52.5 ml
Mint Chocolate bar - MCB	Solid	5	*L. acidophilusnNCFM, L. casei LC-11, B. lactis HNO19*	6.1×10^{9} cells/ serving size	140 g	20 g

The commercial products were tested for the viable counts at three different times during the storage. All the products tested were the freshest found on the grocery store. The products were first analyzed within one week after purchasing, then at a selected mid-point during storage and on the expiration day for the third time (Table 2). The information about the shelf life of each product was obtained by contacting the manufactures by e-mails and over the phone.

Table 2: Age of each product by the time of analysis

Product	Age of the product at reading 1 (Days)	Age of the product at reading 2 (Days)	Age of the product at reading 3 (Shelf life) (Days)
FCW	59	67	75
FCWI	53	101	150
RKWF	12	26	45
FJPB	36	56	75
FDD	58	100	143
MCB	145	210	275

Some products contain a single strain of probiotic while some others contain a mixture of probiotic bacteria. RKWF contains two probiotics and the quantity of each is given separately for a dose. However, other products with a mixture of probiotics do not express the counts separately for each strain (Table 2.1).

Preparation of Samples for Flowcytometry Analysis

Lactobacillus plantarum Lp 400-115B (Danisco, USA) was cultured in MRS broth for 20-24h at 27 ºC. Cells were harvested by centrifuging at 10,000 x g for 3 minutes following the Manufacturer's protocol for Live/Dead® BacLight Bacterial Viability and Counting kit (Molecular Probes, USA). Dead cells were treated with 70%

isopropanol for 45 minutes to one hour according to the manufacturer's protocol. Since this time was not sufficient to kill all bacteria, the time was increased to one and half hours. Live cells were treated with 0.85% NaCl. Both treatment vials were vortexed at every 15 minutes. The well mixed 10^{-1} dilution was filtered through sterile Whatman No. 2V folded filter paper (England) to remove large food matrix particles (Maukonen *et al*, 2006). The filtered samples were pelleted by centrifugation at 10,000 x g for 3 minutes and the pellet was washed twice more than described by the manufacturer's protocol with the diluent to remove matrix particles as much as possible. After washing, the pellet was resuspended in the diluent.

Staining probes in Live/Dead® BacLight test kit (Molecular Probes, USA) are SYTO9 and propidium iodide (PI). The samples and pure cultures were stained as described by the manufacturer. Briefly, for all samples, 977 µl of 0.85% NaCl was mixed with 1.5 µl from each probe in Eppendorf tubes. 10 µl of the 10^{-2} dilution of the sample was then added to the same tube. After staining, the foil covered samples were incubated 15 minutes at room temperature. The stained cells were observed under the Nikon Eclipse Ti inverted microscope with NIS Elements software for the verification of the staining process. Two filters, FITC (green-ET GFP, band pass 525 nm/50m) and TRITC (red-ET dsRed, Band pass 620 nm/60m) from "Chroma Technology" were used to observe the cells. The objective was 60x water immersion objective with 1.2 numerical aperture.

For FCM analysis, 10 µl of the microsphere (Bead solution) was added to stained cell suspension and mixed well by vortexing before the 15 min incubation period. BD FACScan flow cytometer with Cell Quest software was used for analysis of pure culture

and probiotic products. Most samples were analyzed in duplicate in the flowcytometer and some samples were analyzed once per replicate. For setting up the instrument for the experiment four single color controls were used. Forward scatter (FSC), side scatter (SSC) and three fluorescence signals (FL1, FL2 and FL3) were measured. For *Lactobacillus plantarum* pure culture the following detector settings were used. FSC E00 1.00, SSC 335, FL1, FL2 and FL3 705 using logarithmic gains. To correct background scatter the threshold was set at FSC of 54. Gates were made to identify defined populations of the samples in the cytogram of FL1 vs. SSC.

Microbiological Analysis of Commercial Probiotic Samples

Lactobacilli Populations. Ten-fold dilution series from each replicate were prepared by using 0.1% (w/v) peptone water (Difco). For the preparation of the dilution series, 11 ml of liquid products (FCW, FCWI, RKWF, and FJPB) were diluted in 99 ml of 0.1 % peptone water to have 10^{-1} dilution. Five grams of the dairy product (FDD) were suspended in 45 ml of the diluent. For 10^{-1} dilution, each sample with the diluent was shaken vigorously for 30 seconds in sampling bottles. For MCB, 5g were stomached with 45 ml of the diluent in a Stomacher 400 Circulator (Seaward Limited, London, United Kingdom) at 260 rpm for 3 minutes.

For all *Lactobacilli*-containing products, *Lactobacilli* cells were enumerated by pour plating 1 ml portions of the selected dilutions with 15-20 ml of molten MRS agar and incubating at 37 ºC for 48-72 hours anaerobically (BD gas pack system, Becton & Dickinson Corporation, Sparks, MD). For RKWF, to inhibit the yeast growth on the MRS agar, 3 µg/ml of amphotericin were added.

Bifidobacteria Populations. To enumerate *Bifidobacteria*, MRS agar with 0.05% cysteine hydrochloride was used as described in previous studies (Martin *et al*, 2009; Amor *et al*, 2002). Two products (GF and MC) were labeled with both *Bifidobacteria* and LAB. The suitable dilutions were cultured on both MRS agar and MRS agar with 0.05% L-cystein hydrochloride. The inoculated agar plates were incubated at 37 °C for 48-72 hours anaerobically using gas packs (Becton & Dickinson). The plating was performed in duplicate and the colonies were enumerated at the end of the incubation period.

Yeast Populations. Yeast (*Saccharomyces boulardii*, according to the manufacturer) cells were quantified by plating the sample on Y & M (Yeast and Mold, 3M Microbiology Products, St. Paul, MN) petri and incubating at room temperature (25 °C) aerobically for 5 days. RKWF is the only product with a yeast species.

Optimizing Media for Probiotic Enumeration. For MCB and FJPB, the log CFU counts obtained were almost same on both MRS agar and MRS agar with 0.05% L-Cysteinhydrocholride. Cystein decreases the redox potential of the medium and improves the survival of bacteria (Shah, 2000). Product MCB contains the Howaru *Bifidobacterium*, which is *Bifidobacterium lactis* HNO19. Pure freeze-dried cultures of *Bifidobacterium lactis* HNO19 and *L. plantarum* Lp 115-400B were diluted and both strains were plated on both MRS agar and MRS agar with 0.05% L-cysteinhydrochloride to observe the behavior of the two strains on each type of media. When considering the same dilution, *Bifidobacterium lactis* HNO19 did not result in visible colonies on MRS agar, but did so on MRS agar with 0.05% L-Cysteinhydrocholride as expected. *L. plantarum* strain resulted in visible colonies on both types of media making almost same

counts for the same dilution. Therefore, for analysis of products FJPB and MCB, counts were obtained on MRS agar with 0.05% L-cysteinhydrocholride. *Bifidobacterium* species are unable to grow in acidic media where pH is lower than 4.5 and optimum pH is 6.0-7.0 (Rivera-Espinoza and Gallardo-Navarro, 2010; Lourens-Hattingh and Viljoen, 2001). A study was performed by Lahtinen *et al* to assess the survival of *Bifidobacteria* in fermented oat gruel (pH below 4.5) under refrigeration temperature (4 °C) (2005).

Statistical Analysis. Mean and standard deviation for log CFU/g or log CFU/ml were calculated for each product at three different measurement times. The Statistical Analysis System (SAS) version 9.2 (SAS Institute, Cary, NC) was used for analysis. The log conversion of the counts was statistically analyzed using a student's t test as a lower tail test, taking log CFU/ ml or log CFU/g values of the manufacturer's claim and 7 log CFU/ ml or 7 log CFU/g as the reference values. The change of viability and of probiotic bacteria and pH were analyzed by using "PROC MIXED" statement. The level of significance was set at 0.05.

STUDY II

The probiotic culture. The starter culture was freeze-dried *Lactobacillus plantarum* (Lp 115 -400b) pure culture obtained from Danisco (USA). The pure culture contained > 11.60 log CFU/g. The culture was stored in a freezer at -12 °C. The oatmeal food matrix was inoculated to 7 log CFU/g. (The inoculum will be named as *L.plantarum* in the following text.)

Substrate Preparation. The experimental substrates used in the experiment were coconut water (Chaokoh, Thailand) and instant plain oatmeal (Southern Homes).

For convenience and continuous supply, canned coconut water was used. Commercially available coconut water is canned with the pulp. Pulp was removed by filtering. Then the filtered coconut water and oatmeal were sterilized separately for 20 min at 121 °C. The sterile oatmeal was ground in a sterile domestic food processor for two minutes on the 'grind' setting in order to produce a fine powder.

According to the package instructions, the preparation was to mix one packet of oatmeal (28 g) with a ½ cup of water or milk. For the experiment, a ½ cup of coconut water (125 ml) was substituted for the milk or water. The coconut water and fine powdered oatmeal were blended together on the puree setting for one minute.

There were three levels of substrate to be performed under two different temperatures; the control, which is uninnoculated (c), probiotic added (p) and probiotic and prebiotic added (pp) substrate. The food matrix was inoculated in order to be 7 log CFU/g. The control and probiotic added blended mixtures were used to assess the growth rate of *L. plantarum*. The sample size of experiment was three. Samples were analyzed soon after preparation to verify the amount of culturable cells in the inoculum added and during fermentation at each temperature. To monitor the growth at each temperature, samples were taken out at two hour time intervals.

For shelf life analysis C, P and PP were used per each batch (n=3). The inoculum size was the same as for growth rate analysis. PP was added with 1 g of inulin (Alfa Aesar, UK) per 100 ml of the blended P. All preparations and weighing were conducted aseptically.

Fermentation. In order to determine the optimum fermentation temperature, the growth rates of *L. plantarum* were analyzed at three temperatures, i.e. 24, 32, and 47° C. All selected temperatures were close to the optimum growth temperature range of *L. planatarum*, which is 27-30 °C (Derzelle *et al*, 2003; Surono *et al*, 2008). The warmest temperature (47 °C) corresponds to the Donvier yogurt maker (Browne & Co. Ltd, Canada) used in the experiment. The yogurt maker came with plastic containers, which were washed and disinfected with 70% ethanol for sterilization. For the other two temperatures 250 ml Erlenmeyer flasks covered with foil were used. The containers with the inoculated mixture were subjected to three fermentation temperatures. The fermentation was aerobic. There were a control sample and an inoculated sample for each fermentation temperature. For the validation of the process two extra temperatures (27 and 42 °C) were used.

For viability assessment of the oatmeal product during refrigeration storage (n=3), flasks were prepared from each treatment (C, P, PP) as described above. The flasks were fermented for 10h at 27 °C. After the fermentation all experimental units (Earlynmayor flask covered with foil containing the blend of any treatment.) were transferred to the refrigerator at 4 °C. The fermentation was not pH controlled.

Determination of Viability

Growth rate analysis. During fermentation 11 g samples were aseptically removed from each flask and analyzed for viable *L. plantarum* count every 2h to assess the growth rate of the bacterium. The first dilution (10^{-1}) was prepared with 99 ml of 0.1% peptone water in a sampling bottle. The sample bottle was shaken vigorously 30

times. Serial dilutions were prepared using the same 0.1% peptone water. One ml portions from suitable dilutions and 15 ml of molten MRS agar (EMD Chemicals Inc., USA) were added to petri plates in duplicate. Following the pour plate method, the plates with solidified agar were inverted and then packed in anaerobic jars. The plates were anaerobically (BD gas packs) incubated at 37° C for 48-72 hours. The viable *L. plantarum* counts were estimated in colony forming units (CFU)/g. The average of CFU/g for three batches of each treatment was used to obtain data points.

Shelf-life analysis. The first dilution (10^{-1}) was prepared by adding 2 g of the blended mixture with 18 ml of 0.1% peptone water in a sampling bottle weekly. Dilution and incubation conditions were the same as above. The viability *L. plantarum* of the blended mixture was enumerated immediately after the inoculation to determine the inoculum size, after fermentation time (10h) and then as a repeated time analysis for 7 weeks regularly.

pH and acidity analysis. pH measurements for all samples (C, P and PP) were monitored using a standardized Oakton 510 pH meter (OAKTON Instruments, USA) with standard buffers 7 and 4. pH was also measured all the times when the mixture was tested for viability assay.

To measure the titratable acidity, 5 g of the product was mixed with 20 ml of water. The titration was performed with standardized 0.1N NaOH using phenolphthalein as the indicator. Acidity expresses the lactic acid content of the medium. The results were interpreted according to AOAC official methods (1995) for fermented dairy products under section 33.2.06. (1.00 ml of 0.1N NaOH = 0.0090 g of lactic acid).

Rheological Measurements. For rheological measurements, the Brookfield viscometer model HAT was used. Approximately 150 ml of the sample at 24 °C was used in a 150 ml measuring beaker. Spindle number 3 was used at different speed rotations. The rheological parameters for each sample were measured just after inoculation (Day 1) and at the end of the 7 week analysis period (n=3). The viscosity was calculated according to the method used by Mitschka (1982).

Statistical Analysis. Growth rates and Arrhenius activation energy were determined by linear regression analysis. To calculate a theoretical optimum temperature of *L. plantarum* a piecewise linear regression was used to fit the data.

The shelf-life experiment was designed as a split plot block design for repeated measurements. Two factors were considered: prebiotics (applied or not) and storage time. "Probiotics" was the whole plot factor and "prebiotics" was the sub plot factor. Both experimental unit and measurement unit was the flask with the reaction mixture. An analysis of variance was performed for the viability of probiotic bacteria, pH and acidity changes using PROC MIXED on the Statistical Analysis System (SAS) version 9.2 (SAS Institute, Cary, NC). The level of significance was set at 0.05. The experiment was done in triplicates (n=3).

RESULTS AND DISCUSSION

STUDY I

Fluorescent Staining and Flowcytometry Analysis of Commercial Probiotic Products

Flowcytometry (FCM) is used to detect the subpopulations of viable but non culturable, damaged or dormant cells in a sample (Diaz, 2010). The dead cells were observed in green with the FITC (green) filter and live cells were red with the TRITC (Red) filter, when observed under the fluorescent microscope. Changing the incubation period, diluting the two dyes and sample was not effective. The stained matrix particles were also observed brightly. The instrument was calibrated by single stained live and dead controls with both dyes separately. Using double stained live and dead cells the gates were created to identify the dead and live bacteria populations on cytograms. Gates are graphical boundaries that sort interested populations for further analysis. Cytograms of test samples gave the gate statistics which gives the number of events through the gates created (Figure 3).

The use of FCM analysis to count the viable cells was found to be unreliable for the conditions used in the experiment, especially due to the presence of debris even in the filtered samples.

FCW, RKWF and FJPB products possess FCM counts for initial counting and intermediate counting. Though lack of enough data for FCM analysis prevents drawing many conclusions, available data do not always give higher counts than plate counts. The loss of viability of bacteria during storage is strain specific and this does not result in

significant differences in counts in FCM and plating (Kramer, 2009). Products RKWF and FCWI have reported higher counts than the plate counts in FCM analysis. This could be due to over estimation of the actual probiotic count. When there are numerous matrix particles with a similar size of probiotic cells and those particles are brightly stained as bacteria, the resulting counts could be higher than the actual count. All other products obtained higher counts by plating. Studies by Maukonen *et al* in 2006 have also reported obtaining higher counts by plating over fluorescent techniques because some cells may exist as clumps resulting in lower counts in flowcytometry than in plate counting. Not having strong evidence to prove the hypothesis on presence of viable but non-culturable cells (VBNC) cells and high instrumentation cost persuaded us to give up FCM analysis.

Microbiological Analysis for Commercial Products. Commercial probiotic products are marketed emphasizing the viable cell number in the product. Hence, consumers expect to have claimed live counts of probiotics by the manufacturer at the end of shelf life of a product. Table 3 shows the obtained plate counts of the tested commercial products during three stages of the shelf life of each.

Preliminary studies showed that taking three samples from each product was sufficient to assess the viability of products. There were at least 2 replicates for each sample in plating.

Figure 3: A & B - The gates created for double stained live and dead cells, C - The cytogram for one of the FCW samples, the isolated population is for beads (microsphere solution)

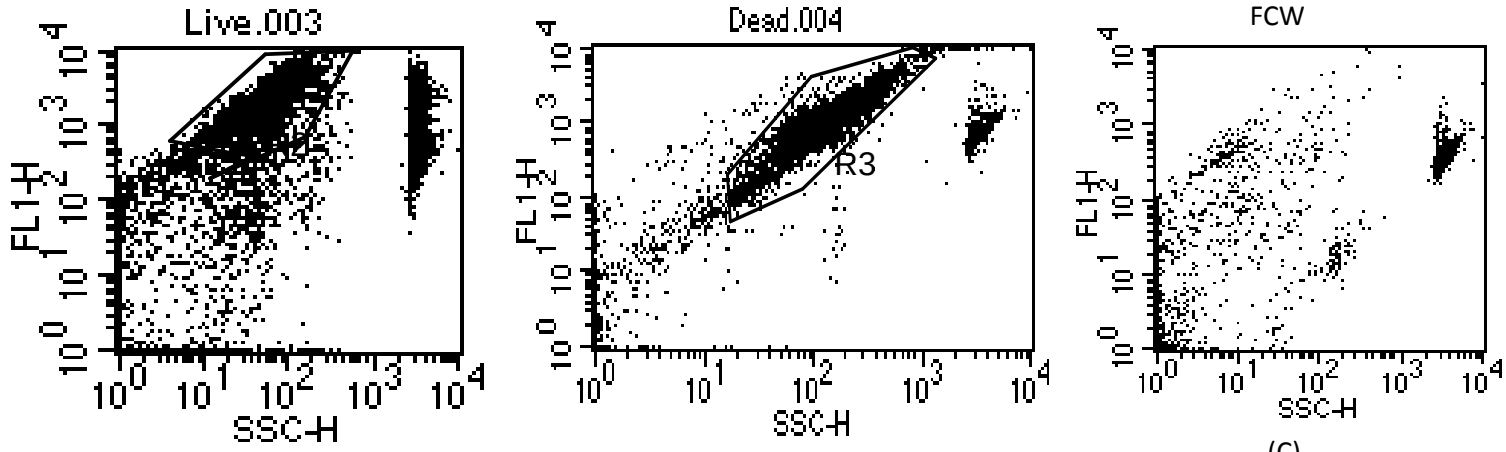


Table 3: Comparison of counts (log CFU/ml or CFU/g) obtained by plate counting [a]

NC- Not claimed

Product	Number on the label (log CFU/ml or CFU/ g)	Initial Analysis		Mid-shelf life Analysis		The end of shelf life analysis	
		Age of the product (Days)	Average count (log CFU/ml or CFU/g)	Age of the product (Days)	Average count at (log CFU/ml or CFU/g for)	Age of the product (Days)	Average count at (log CFU/ml or CFU/g)
FCW	7.32_{A} [b]	59	$8.49 \pm 0.31_{A}$	67	$8.49 \pm 0.14_{A}$	75	$8.06 \pm 0.13_{B}$
FCWI	7.62_{A}	53	$6.71 \pm 0.13_{B}$	101	$6.14 \pm 0.11_{B}$	150	$6.24 \pm 0.04_{B}$
RKWF	6.92_{A}	12	$6.61 \pm 0.15_{B}$	26	$6.12 \pm 0.48_{BC}$	45	$6.09 \pm 0.15_{C}$
FJPB	NC	36	$8.55 \pm 0.01_{A}$	56	$8.53 \pm 0.16_{A}$	75	$8.52 \pm 0.17_{A}$
FDD	8.68_{A}	58	$9.06 \pm 0.07_{A}$	100	$7.61 \pm 0.21_{B}$	143	$7.12 \pm 0.05_{B}$
MCB	8.48_{A}	145	$7.90 \pm 0.14_{B}$	210	$8.06 \pm 0.20_{B}$	275	$7.91 \pm 0.13_{B}$

NC – Not claimed

[a] Results for all products are summarized by means ±standard deviation for 6 products, tested at three different times during refrigeration storage in duplicate

[b] Means within a given row with the same letter are not statistically different from each other (α=0.05)

According to the Table 3, all the products, except FCW, show lower counts at the end of shelf life than the claimed numbers in the labels. Consequently, the companies are not complying with their own claims always. Generally, probiotic products are recommended to be stored under refrigeration temperature. Though the FCW has a higher count than the claimed count, storage has a significant effect on viable count ($p<0.05$) at the end of the shelf life of 75 days. FCWI is also the same probiotic carrier as well as FCW. But, the first count, which was taken at 53^{rd} day of the shelf life was significantly lower than the claimed count ($p<0.05$).

Due to being an unpasteurized product, RKWF has the shortest shelf life among the all samples. The manufacturer claim is 6.92 log CFU/ml and the counts obtained by plate counting (Table 3) through the shelf life (45 days) were significantly lower than the manufacturer's claim ($p<0.05$).

FJPB does not have a claim for the probiotic population. Therefore, the counts were compared to the 10^7 CFU/ml, which is considered as the probiotic count to be acceptable for being functional. As shown in Table 3, counts obtained were fairly constant during shelf life of 75 days under refrigeration storage. Though the viability is lost for some probiotics in fruit juices during storage (Sheehan, 2007), FJPB; the only fruit juice among the selected products does not show a significant variation of the viable counts.

FDD, a dairy substrate appears to be an ideal food matrix to maintain viable probiotics, the counts obtained by plate counting were expected to comply with manufacturer claim. However, plate counts decreased from 9.06 log CFU/ ml to 7.12 log

CFU/ ml by the expiration day (143 days). Statistical analysis shows the manufacturer claim is not acceptable ($p<0.05$) at the end of the shelf life. Storage at refrigeration temperatures has an obvious influence on the reduction of viable counts ($p<0.05$) over the time.

For MCB the counts were significantly lower than the manufacturer claim (8.5 log CFU/g) in all shelf life times tested (Table 3). When compared to all products MCB is the most stable product over the storage during the shelf life. High content of probiotic CFUs in a food item does not always mean the counts reach the intestines are high. Consequently, though the obtained counts for MCB are lower than the manufacturer claim, compared to the obtained stability of the counts and findings by Possemiers in 2010, high viable cell counts may reach the GI tract.

According to the analysis of counts, products FCW, RKWF and FDD show significant reductions of viable counts and viable count changes for FJPB, MCB and FCWI are not significant during the shelf life of each product at 4 °C. The rate of losing viability for probiotic cells of FDD, FCW and RKWF are -1.4×10^7 CFU/ day, -1.6×10^7 CFU/ day and -9×10^4 CFU/ day respectively within the test duration at 4 °C.

Obtaining same results on MRS and MRS with cystein-hydrochloride would be due to lack of culturable *Bifidobacteria* cells. But this cannot be verified without identification of colonies and not knowing the added inoculum size by the manufacturer. The available results with Live/Dead FCM assay did not support the argument.

Changes of pH of the products. pH is considered as the logarithmic value of the reciprocal of the hydrogen ion concentration in a medium. The oatmeal – coconut water food matrix was supposed to be acidic due to production of lactic acid by *L. plantarum* by homofermentation and the acidity of the medium would be changed consequently. In refrigeration storage, pH was expected to be constant and not in among detrimental pH values for the survival of probiotics. There were slight pH changes during the refrigeration storage. However, the overall pH changes for all products during the specific shelf lives are not significantly different at the end of the shelf life.

For FJPB and FCWI, the pH changed 0.1 units and all others were less than 0.1. FJPB and FCW contain *L.plantarum* 299v. The pH values of the products at the end of shelf lives were 3.76 and 4.15 respectively. But the survival is very high for both products. According to Molin in 2001, *L.plantarum* 299v is highly resistant to low pH of fruit drinks and the viability is very high at the refrigeration temperature for more than one month. Previous experiments have also observed an unchanged pH value over the storage at 4 °C for fruit drinks (Champagne, 2008). Only product FDD has an increased pH on the expiration date. But this increment for 5 samples, is not significant ($p>0.05$) statistically (Table 4).

Table 4: pH changes of the liquid commercial probiotic products over the storage time in refrigerator (4 ºC)[a]

Product	Storage time at initial reading (Days)	Initial pH ± SD	Storage time of each product by the end of the shelf life (Days)	Final pH ± SD
FCW	3	$4.20 \pm 0.05_A$[b]	16	$4.15 \pm 0.03_A$
FCWI	2	$3.98 \pm 0.10_A$	97	$3.87 \pm 0.15_A$
RKWF	3	$3.49 \pm 0.07_A$	33	$3.42 \pm 0.04_A$
FJPB	3	$3.87 \pm 0.07_A$	39	$3.76 \pm 0.13_A$
FDD	4	$3.92 \pm 0.01_A$	85	$3.97 \pm 0.00_A$

[a] Results for all products are summarized by means ±standard deviation for 5 liquid products, tested at two different times

[b] Means within a given row with the same letter are not statistically different from each other (α=0.05)

Selected products have different shelf lives. Manufacturers' claims for CFUs on labels are different from product to product. Label of FCW expresses the probiotic count at the time of production, but not at the end of the shelf life. Some manufacturers guarantee the probiotic counts till the end of stated shelf life (FDD, MCB).

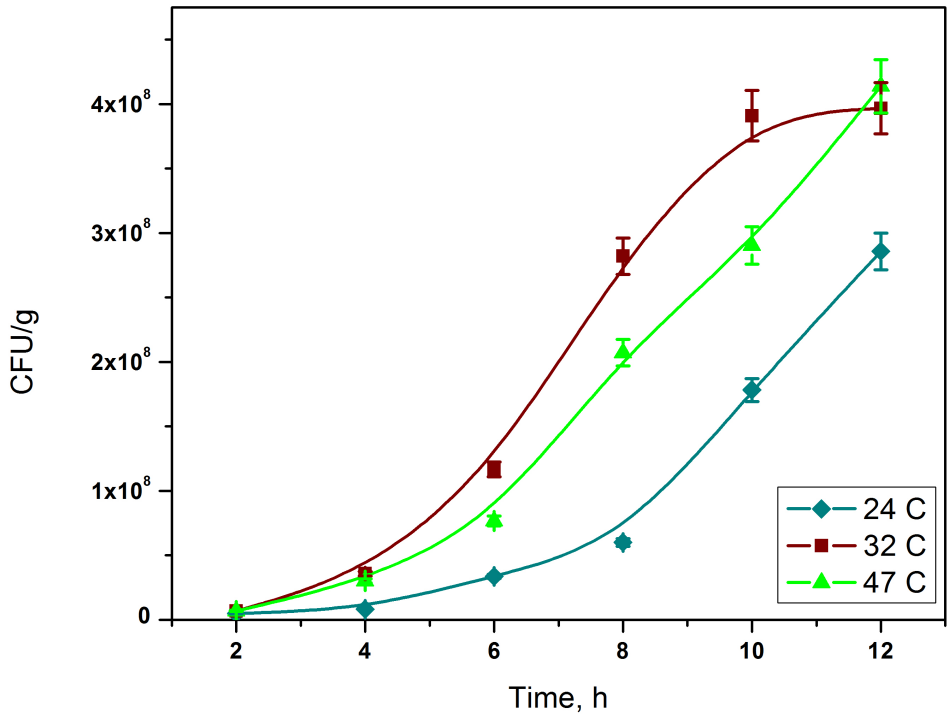
The guidelines for probiotic product labeling require the genus, species and strain, the live cell count at the end of shelf life, serving size, which is sufficient to serve therapeutic minimum, health claims, storage conditions and contact details of manufacturer (FAO/WHO, 2002). Our study indicates that there were some deviations from the guidelines by not providing the species and strains (RKWF) and viable count at the end of shelf life (FJPB, FCW).

STUDY II

Influence of Temperature on the Growth of *L. plantarum.*

Growth curves for *L. plantarum* at slected temperatures are shown in Figure 4. The growth at 32 °C represents the characteristic sigmoid shape of the growth cycle. Lower and higher temperatures from the optimum temperature of *L. plantarum* show an elongated log phase. At 24 °C and 32 °C, there was a lag phase of 2h and at 47 °C lag phase was around 4h.

Figure 4: Growth of *L. plantarum* over 12 h period for selected fermentation temperatures

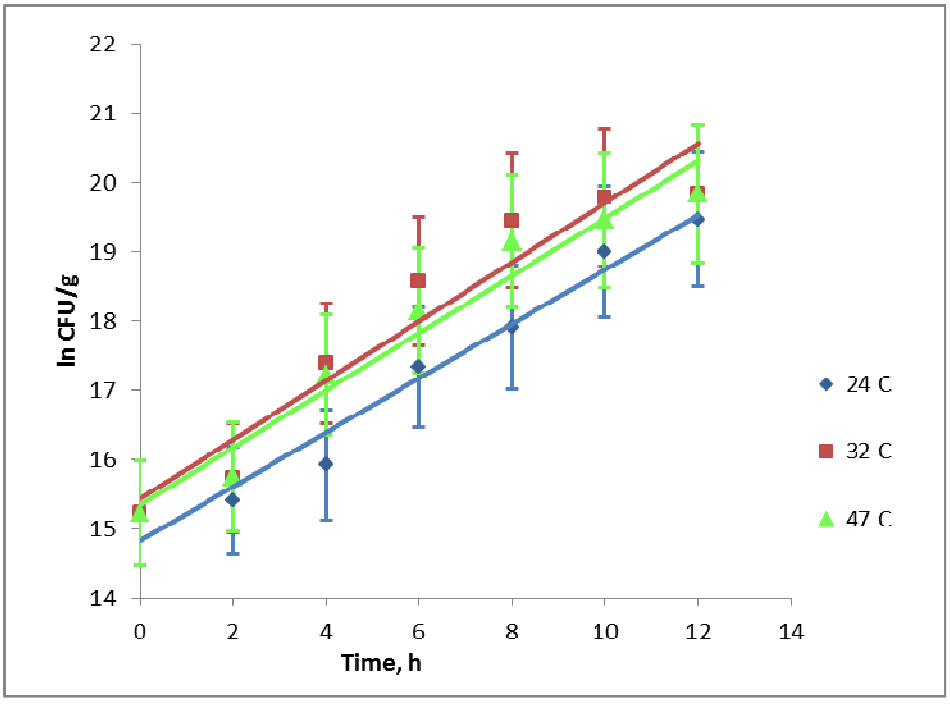


It seems *L.plantarum* has reached its stationary phase within 10h of fermentation at 32 °C and when the growth temperatures deviated from the optimal range, the time spent in the exponential phase is longer, but the growth rate is lower. Similar results have

been reported in a cereal fermentation study done with one *L.plantarum* strain by Charalampopoulos et al (2002). When wheat, malt and barley were inoculated with *L. plantarum*, the exponential growth has been observed until 10–12 h of fermentation at 37 °C.

As expected, the growth rate was higher at 32 °C (Figure 5), which is the closest temperature to the optimal growth temperature of *L. plantarum*. The specific growth rate at 32 °C is 0.5075/h. The specific growth rates of *L. plantarum* at the coldest temperature selected (24 °C) and the warmest temperature (47 °C), were lower than that of 32 °C.

Figure 5: The effect fermentation temperatures on the growth rate of *Lactobacillus plantarum*



Though mesophillic *Lactobacillus plantarum* does not show a growth at 45 °C (Wheater, 1955), a moderate growth was observed in this experiment when the yogurt maker was used (47 °C).

To confirm the effect of temperature on the growth rate, two other fermentation batches were set up; first one at 27 °C and the second one at 42 °C (Figure 7). The expectation was to observe intermediate growth rates; between 24 and 32 ºC for 27 ºC and between 32 and 47 ºC for 42 ºC.

Though the yogurt maker showed a good growth pattern at 47 ºC, the confirmation batch fermentation temperature of 42 ºC does not show a growth with a steep exponential phase. However, at 27 ºC the expected "sigmoid shaped growth" was obtained. Growth rates were calculated using Figure 8 and the corresponding growth rate constants (k) are included in Table 5 with a comparison of the growth rates at other fermentation temperatures.

Figure 6: The growth of *Lactobacillus plantarum* at selected intermediate fermentation temperatures

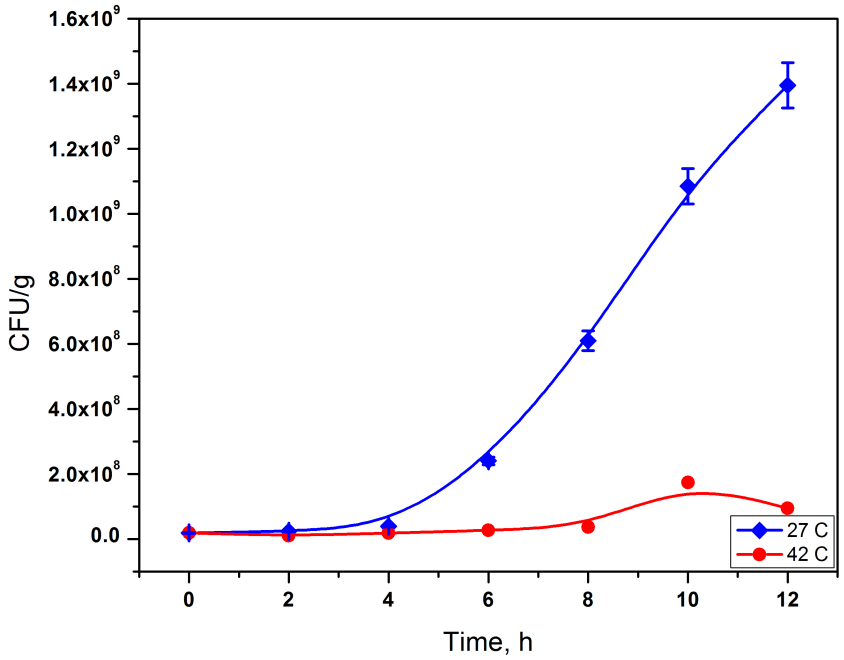


Figure 7: Growth rate determination of *L. plantarum* at intermediate temperatures

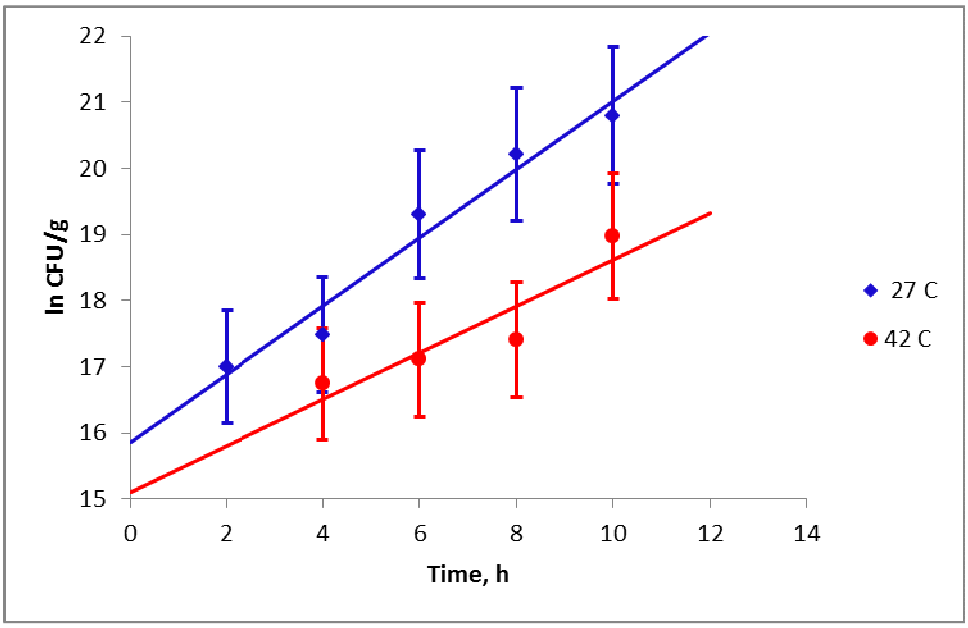


Table 5: The specific growth rates of *Lactobacillus plantarum* at selected fermentation temperatures

Temperature ºC	Temperature K	k (h^{-1})	R^2
24	297	0.391	0.935
27	300	0.516	0.867
32	305	0.502	0.964
42	315	0.351	0.937
47	320	0.414	0.982

The Arrhenius activation energy Ea calculated by Figure 8 slopes indicates a switch in value and direction. For the warmer temperature range (32-47 ºC), the E_a value is 13.2 $kJmol^{-1}$, while at the cooler temperature range (24-32 ºC), the E_a value is -20.72 $kJmol^{-1}$.

Activation energy of a microorganism reveals the temperature sensitivity for the growth rate of that microorganism (Andre´s *et al*, 2004). At colder temperatures E_a takes

a negative value while it is positive at higher temperatures. The negative E_a means that the growth occurs well using the available substrate well. Positive E_a means that the growth is retarding. The integrated effect of colder and warmer temperatures can be used to calculate the optimum theoretical growth temperature of the experiment (Figure 9). The intersecting times cross at 30 ºC, which is within the optimal growth temperature range of *L. plantarum*. Figure 3.8 confirms the difference between a biological system and a chemical system. Our experiment obtained the highest growth rate at the temperature of 27 °C (k=0.51 h^{-1}). The graphical calculation of optimal growth temperature is 30 °C. This calculation fits well to the experimental design by De Angelis *et al* (2004), who demonstrated the heat shock responses of *L. plantarum* considering the optimum growth temperature as 30 ºC.

Figure 8: Effect of temperature on probiotic fermentation. A- warm temperature range: 32-47 ºC, B- cold temperature range: 24-32 ºC.

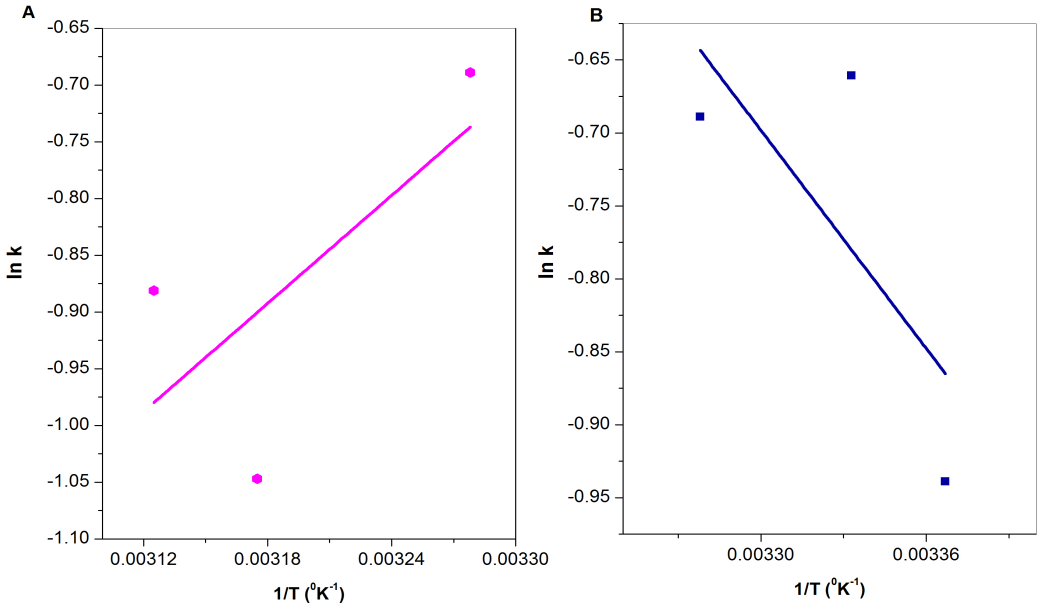
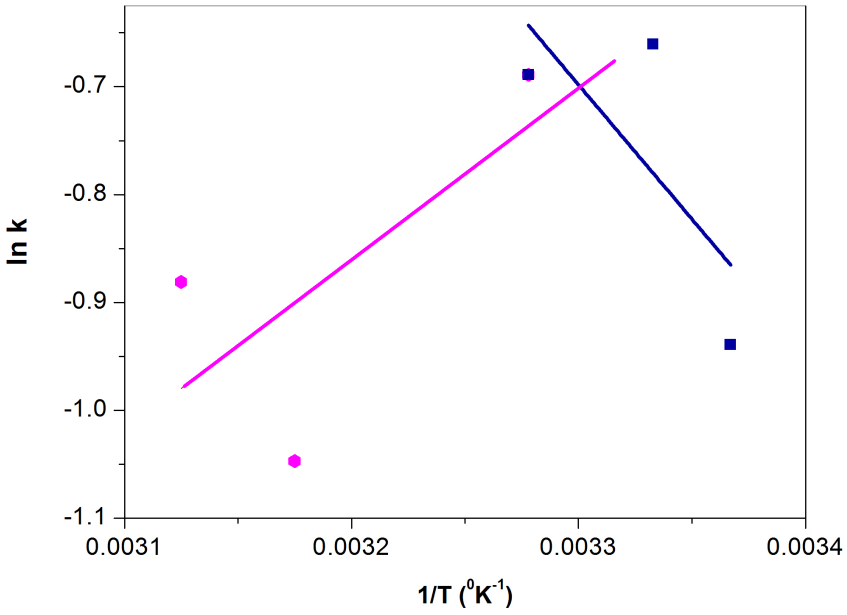


Figure 9: Graphical illustration of the optimum growth temperature of *L.plantarum* during probiotic fermentation



Viability Analysis during the Refrigeration Storage of Probiotic Fermented Oatmeal Batches

Viability of *L. plantarum* in the oatmeal-coconut water mixture was monitored to measure the probiotic potential of the substrates. Three batches of fermented coconut water - oatmeal were used to determine the survival of the probiotics during refrigeration storage (Table 6). Each batch contained one P and one PP. The probiotic cells were enumerated soon after the inoculation. The initial probiotic population increased in more than 1 log after the first eight days in the refrigeration storage. The probiotic population for each batch was $\approx 10^7$ CFU/g at the end of 49 day refrigeration storage (Table 6).

The corresponding p values for statistical analysis of factors influencing the viable count, show that the addition prebiotics and refrigeration storage time (day) have

significant effects with $p<0.05$. Refrigeration storage, makes significant differences in viable counts ($p<0.05$) between the P and PP. The differences of least square means points out the significance of each factor throughout the repeated analysis. Addition of prebiotics does not show a significant difference till day 14 and afterwards the growth retardation in inulin added samples is significant with $p < 0.05$.

According to the findings of Charalampopoulos *et al*, under natural conditions using 10^7 CFU/ml of viable probiotic cells with cereals gives enough space for the growth of probiotic strains preventing the growth of undesired microorganisms in the cereal matrix (2002). This probiotic strength increases the product safe. Following these findings, the viability was monitored until the probiotic cell count reached 10^7 CFU/g in this study.

Table 6: The average of probiotic log counts (n=3) of oatmeal-coconut water matrix during refrigeration storage (4 ºC)

Time (Days)	**Log CFU/g (P)**	**Log CFU/g (PP)**
0	7.06± 0.27	6.99 ± 0.27
0.42	8.55 ± 0.11	8.09 ± 0.16
7	9.12 ±0.01	9.01 ± 0.11
14	8.89 ±0.02	8.75 ± 0.04
21	8.37 ± 0.22	7.97 ± 0.15
28	8.01 ± 0.15	7.46 ± 0.03
35	7.75 ± 0.15	7.05 ± 0.08
42	7.54 ± 0.10	6.66 ± 0.07
49	7.23 ± 0.02	6.41 ± 0.06

Different studies have been done on the inulin consumption by *L. planatarum* strains. The results are controversial. Both growth enhancing effects and growth retarding effects have been observed. *L. planatarum* No 14 has shown that inulin supports the

growth in *in-vitro* studies. Also the growth of the same strain was higher when mice were fed with an inulin diet. This evidences that inulin supports the in-vivo growth of *L. planatarum* no14 in the mouse gut (Takemura *et al*, 2010). One *L. planatarum* strain isolated from white cheese did not ferment inulin at all but was able to ferment fructooligosaccharides (FOS) and galactooligosaccharides (GOS) (Georgieva *et al*, 2009). In 2009, Kalui *et al* screened 20 *L. planarum* and *L. rhamnosus* strains for the fermentability of inulin by culturing the strains on 2 % inulin added MRS agar. Out of 20 strains 2, *L. plantarum* strains utilized inulin.

L. plantarum can also utilize the prebiotics substrates present in oatmeal. In a study conducted to investigate the consumption of oat bran components such as xylo-oligosaccharides, only *L. plantarum* was able to proliferate, when compared to *Lactococcus lactis* and *L. rhamnosus* (Kontula *et al,* 1998). According to the studies by Molin, *L. plantarum* 299v fermented oatmeal gruel with fruit juices has increased the carboxylic acid (SCFA) concentration of the feces in healthy individuals (2001). This has been increased SCFA concentration in lumen, which is beneficial for the status of mucosa.

pH changes of Oatmeal-Coconut Water Matrix.

The oatmeal does not contain any sugars. Sucrose addition to the oatmeal mash decreases the fermentation time and pH and added carbohydrates have yielded better counts (Angelov *et al*, 2006). However in our study, the added sugar of coconut water did not drop the pH of the medium less than 4.5 (Table 7).

In this study storage influences in the changes of pH significantly ($p<0.05$). The differences of least square means confirms that the final pH of product proves that the effect due to prebiotics is not significant in determining the pH of the product ($p>0.05$). Under both fermentation conditions, final pH values are significantly different from the initial pH with $p <0.05$.

Table 7: The changes of average pH values (n=3) of oatmeal-coconut water matrix during refrigeration storage (4 ºC)[a]

Time (Days)	pH of P	pH of PP	pH of C
0	5.78_{A}[b]	5.75_{A}	5.79_{A}
0.42	5.33_{B}	5.19_{B}	6.04_{B}
7	5.23_{C}	5.08_{B}	6.22_{B}
14	5.18_{C}	5.04_{B}	6.23_{B}
21	5.20_{C}	5.07_{B}	6.24_{B}
28	5.21_{C}	5.08_{B}	6.25_{B}
35	5.22_{C}	5.02_{B}	6.17_{B}
42	5.45_{D}	5.15_{B}	6.21_{B}
49	5.43_{D}	5.12_{B}	6.24_{B}

[a] Results for all treatments are summarized by means tested at two different times during refrigeration storage

[b] Means within a given column with the same letter are not statistically different from each other ($\alpha=0.05$)

pH of the food matrix is considered as a critical factor that determines the stability of probiotics during storage (Champagne and Gardner, 2005). Due to the metabolism of the LAB the medium becomes more acidic and thus the pH of the medium decreases. The probiotics should be able to survive under such acidic environments and the cultures should be functional during storage. The bacteria in higher acidic condition are more tenacious and last longer during the shelf life of the product. “Over acidification” or post – production acidification” is due to a further drop of pH of the product after

fermentation and during refrigerated storage (Lourens-Hattingh and Viljoen, 2001). But this can be prevented by good manufacturing practices to control the growth of the cultures and using cultures with reduced acidification effect (Lourens-Hattingh and Viljoen, 2001).

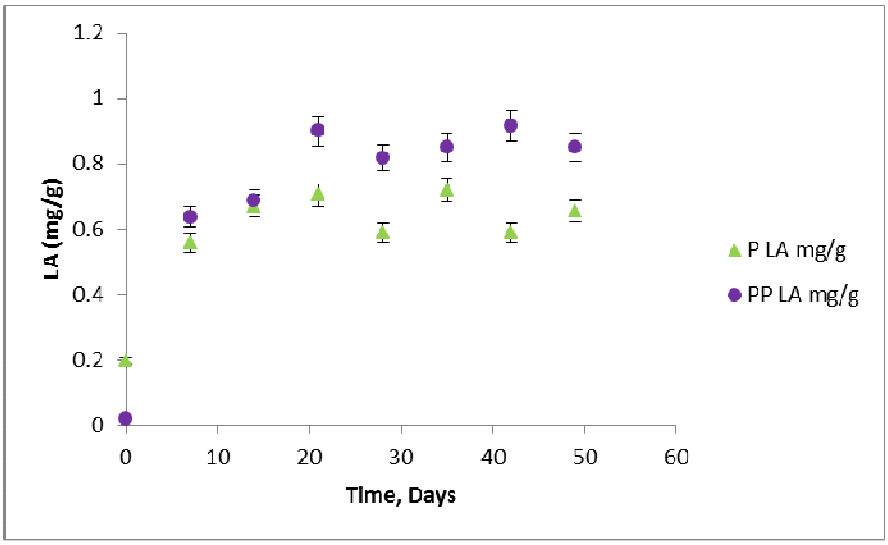
According to Giruad *et al* in 1991, the optimum pH for *L. plantarum* is 6.0, and the bacterium grows at 3.4-8.8 range of pH. In determining the viability, having a pH above 4.0 during the storage for a fermented beverage is recommended (Angelov *et al*, 2006; Gupta *et al*, 2010). Although there are not clear regulations about the pH of fermented functional foods, in most countries yogurt should have a pH below 4.5.

The pH of the medium is one of the intrinsic factors that determines the microbial growth. pH values beyond the growth pH range may retard the bacterial growth changing the intracellular pH. During fermentation, high acidity may denature the proteins including the enzymes and hence, changes of cell membrane permeability, DNA replication, ATP synthesis, RNA synthesis etc. may be affected. For growth rate analysis, at all fermentation temperatures pH did not reach a growth limiting value. The oatmeal - coconut water mix does not contain much sugar and there is not added sugar during preparation. According to Yong *et al* (2009), the sugar content of fresh young coconut/ green coconut water is around 2.61g/100g. The commercial coconut water used in our experiments contains 11.43g sugars/150 ml of coconut water.

Production of acidic metabolites drops the final pH. Addition of prebiotics did not significantly influence the final pH of the product, when compared to the influence of the storage time ($p>0.05$).

The acidity was expressed as mg (lactic acid) / g (product) (Figure 10).

Figure 10: The changes of acidity for fermentation with the effect of prebiotics



Oatmeal itself contains calcium carbonate ($CaCO_3$) as a source of calcium. $CaCO_3$ is used to increase the pH of soil and microbial growth media. The food matrix is slightly acidic after the microbial fermentation. Lactic acid, which is a weak organic acid produced during bacterial fermentation dissociates incompletely resulting in H^+. These Hydrogen ions react with CO_3^{2-} and form HCO_3^- ions and the calcium salt of the weak acid is made.

$$R\text{-}COOH + CaCO_3 \longrightarrow R\text{-}COO\text{-}Ca + HCO_3^-$$

It is assumed that under refrigeration temperatures, fermentation does not progress and weak organic acids formed during the fermentation get neutralized, increasing the pH of the medium (Table 7).

Viscosity Analysis

Rheological parameter are good indicators of texture and important for consumer acceptance. According to findings of Martensson *et al* in 2000, the EPS production by some LAB strains is dependent on fermentation time and temperature. The viscosity of the novel food matrix was monitored to observe the production of EPS by *L. plantarum*. The rheological analysis of the product shows the curve of the shear stress-shear rate plot begins at the origin of the plot and concaves upwardly. Therefore the increment of shear rate is not proportional to the increment of the shear stress showing a non-Newtonian fluid with a pseudoplastic/shear thinning behavior. The shear thinning behavior was observed both on production date and on the expiration date (Figure 11).

The flow behavior index (n) that should be <1 for a shear thinning fluid was observed on both analysis days (Table 8). The apparent viscosity was calculated at each shear rate, and the plot apparent viscosity vs. shear rate shows that the apparent viscosity decreases with the increasing shearing rate.

Figure 11: The plots of average shear stress vs. average shear rate for three oatmeal-coconut water matrices on the production date (Day 0) and on expiration date (Day 49)

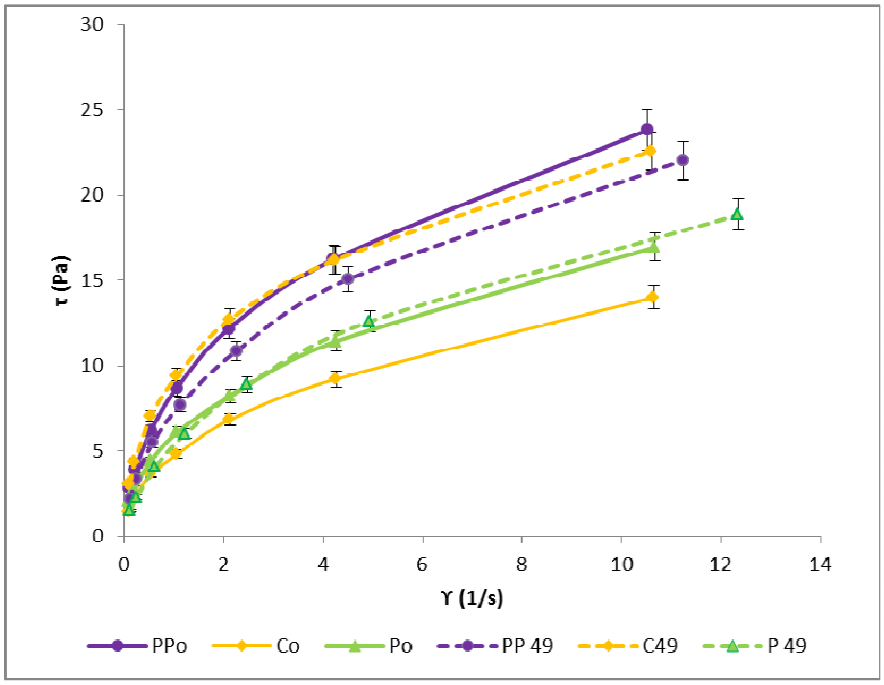


Table 8: The changes of the flow index behavior (n) of oatmeal-coconut water matrix during the shelf life[a]

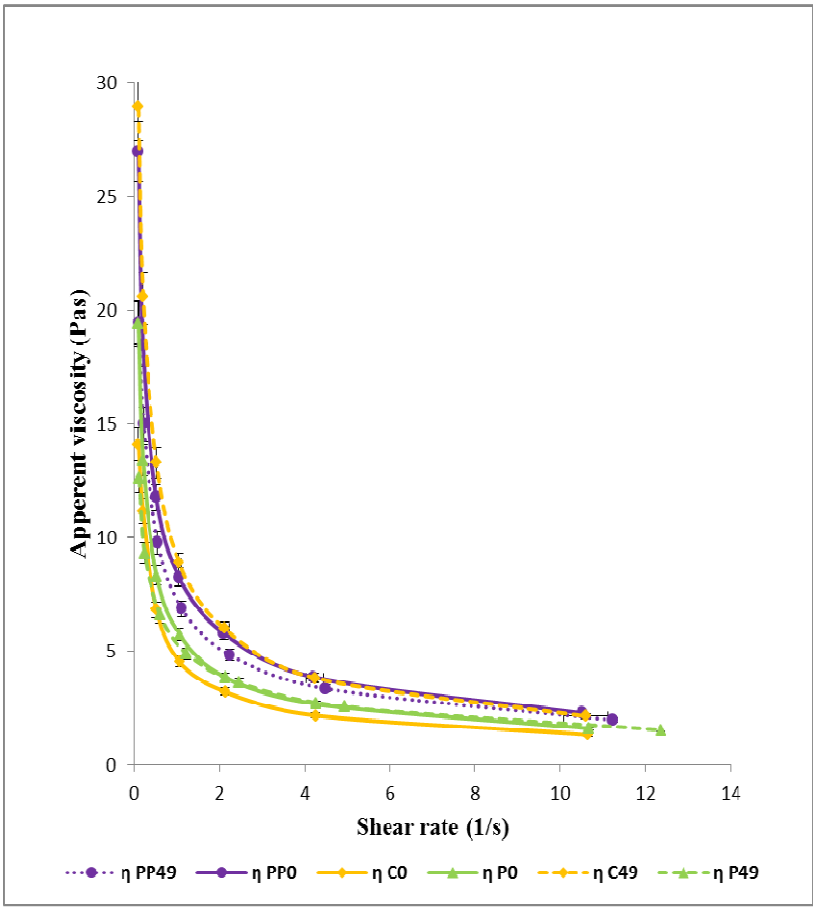
Sample	Flow index behavior (n) on the production date	Flow index behavior (n) on the expiration date
C	$0.47 \pm 0.01_{A}$[b]	$0.46 \pm 0.04_{A}$
P	$0.46 \pm 0.02_{A}$	$0.50 \pm 0.06_{A}$
PP	$0.46 \pm 0.01_{A}$	$0.51 \pm 0.03_{A}$

[a] Results for all treatments are summarized by means ±standard deviation tested on production date and expiration date

[b] Means within a given row with the same letter are not statistically different from each other (α=0.05)

As the figure 12 shows, addition of prebiotics or storage time does not contribute significantly on changes of apparent viscosity ($p > 0.05$).

Figure 12: The plots of changes of apparent viscosity with the rising shear rate for the production date and expiration date



Previous studies have shown *L. plantarum* is an exopolysaccharide (EPS) producing probiotic (Tallon *et al*, 2003). As suggested by Martensson *et al* in 2000, the EPS production can be monitored by viscosity measurements. In a previous study of fermenting an oat based nondairy milk substitute with 9 mesophilic LAB strains resulted in 3.6-5.1 final pH. As the proteins in oat do not possess a tendency of coagulating at this pH range, viscosity measurements are appropriate to identify the EPS production. The ideal pH for EPS production is 6.2 (de Vuyst *et al*, 1998). In this study the samples

containing the active probiotics (P and PP) always had a pH lower than 6.2. However, it was always higher than pH 5.0. Hence, the protein coagulation cannot be expected and the viscosity changes in P could be due to EPS production by *L.plantarum*. But the data does not provide a significant change of apparent viscosity, which was expected to be due to EPS production.

β glucan of oats adds some viscous nature after dissolving in water. Commercial oatmeal contains guar gum, which improves the stability of the product by preserving moisture. Guar gum also has a shear thinning/pseudoplastic behavior. But, the effect of these components is not strong enough to change the apparent viscosity of the all treatments significantly ($p > 0.05$).

CONCLUSIONS

STUDY I

In conclusion, our study shows that the manufacturer claim on the label is not always accurate. In this study all the products tested reported good viability through plate counting. Though all products did not meet manufacturer claim, still the products had acceptable counts (in the range of 6-8 log CFU/ml or CFU/g). However, our objective was testing the accuracy of manufacturers' claims. Out of the products with a claim on the label, only fermented coconut water (FCW) maintained the claimed viable count during the refrigeration storage. Nevertheless, the highest deactivation rate was also observed in fermented coconut water.

Though plate counting is time consuming, it always gave acceptable counts compared to flowcytometry. Flowcytometry results are highly dependent on staining, which was not efficient in this study with Live/Dead test kit. The cell suspension with food particles may be interfering the actual counts. Plate counts taken at three different times through the shelf life of each product, express that mint chocolate bar (MCB) and fruit juice with pomegranate and blackberry flavor (FJPB) have more stable counts over refrigeration storage. Therefore, according to the study chocolate and fruit juices are the best matrices for carrying probiotics.

The pH change during the storage time is not significant for all tested probiotic products.

STUDY II

For fermentation, the optimum temperature from literature, 27 ºC was used and the exponential growth was observed from 2-10h period. Fermentation at the optimum growth temperature of starter culture gives better results regarding viability. The growth rate analysis studies prove the theoretical optimum temperature of *L. plantarum* (Lp 115-400B) is 30 °C, which falls in optimum temperature range. Adding some prebiotics to the probiotic containing matrix adds symbiotic qualities for the food matrix. In this study, the minimum documented effective dose of inulin was used (1g). At the end of evaluation time there was not a significant difference in CFU/g between fermentations with or without inulin. This would be due to two reasons. Either this strain of probiotics does not ferment the prebiotic inulin or the used dose is insufficient to induce a positive growth. Though adding inulin was not effective towards the cell count over the time it may increase the gut health due to the bifidogenic activity. Under the prevailing conditions inulin is not effective for the improvement of growth of *L. plantarum* (Lp 115-400B) in the used food matrix.

In all trials, the inoculum size was 7 log CFU/g of probiotics to the novel matrix. The first sample was taken soon after the inoculation. Therefore, under processing conditions, the viability of the cells may be unharmed.

pH of the product was higher than the critical 4.5 value and the storage time had a significant influence on changes of the pH. Addition of prebiotics did not significantly lower the pH.

The novel food matrix had a pseudoplastic rheological behavior. Though an enhanced viscosity was expected at the end of the storage due to microbial exopolysaccharide production, the apparent viscosity changes were not significant at the end of storage for any treatment.

Our studies demonstrate that the viability of *L. plantarum* (Lp 115-400B) in coconut water was improved by adding oatmeal. This would be an ideal home-made probiotic product for the regular consumption of probiotics for general public.

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